Pharmaceutically active compounds might bioaccumulate and cause effects on the free – floating macrophyte *Ceratophyllum demersum*

D i s s e r t a t i o n

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Ein Gelehrter in seinem Laboratorium ist nicht nur ein Techniker; er steht auch vor den Naturgesetzen wie ein Kind vor der Märchenwelt

~Marie Curie~

Inhalt

I. Abbreviations

ANOVA	analysis of variance
ATP	adenosine triphosphate
BCF	bioconcentration factor
BMAA	β-N-Methylamino-L-alanine
C	control treatment
Ca	calcium
CAT	catalase
Cd	cadmium
CDNB	1-chloro-2,4-dinitrobenzene
Cr	chromium
Cu	copper
CYP450	cytochrome P450 monooxygenase
d	day
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DNAse	desoxyribonuclease
DTE	dithioerythrit
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
Fe	iron
FW	fresh weight
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	glutathione disulfide
GST	glutathione S-transferase
h	hour
HCH	hexachlorocyclohexane
HPLC	high performance liquid chromatography
K	potassium
kat	katal
L	liter
LC-MS/MS	liquid-chromatography tandem mass spectrometry
M	molar
MeOH	methanol
Mg	magnesium
mg	milligram
mGST	membrane bound GST
min	minute
mM	milimolar
Mn	manganese
MT	metallothionein
Na	natrium
NADH	nicotinamide adenine dinucleotide (reduced)
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NaP	sodium phosphate

NAP-10	Sephadex G-25 column
NAP-5	Sephadex G-25 column
nkat	nano katal
OECD	organization for economic co-operation and development
Pb	lead
POD	peroxidase
PQ	plastoquinone
PS	photosynthesis
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcriptase
RNZIH	Royal New Zealand Institute of Horticulture
SD	standard deviation
sGST	soluble GST
STP	sewage treatment plants
TCA	trichloroacetic acid
TRIS	tris (hydroxymethyl) aminomethane
Temp	temperature
U	units
ww	wet weight
xg	x gravity
Zn	zinc

II. Summary

Pharmaceutically active compounds (PhACs) are detected, ubiquitously in physiologically relevant amounts (> 1µg/l), in all anthropogenic impacted surface waters. In urban used areas the surface waters are impacted through primarily anthropogenic usage (from household or clinical medicine wastewater, veterinary application, etc.). More than 80 different PhACs and their metabolites are detected in aquatic ecosystems, including freshwater, marine water and the sediment. PhACs from human medicine or veterinary reach the environment primarily through sewage treatment plants (STP).

The concentration of therapeutically used PhACs are during the process of STP usage reduced, through microbial degradation or absorption of the substances in the sewage sludge, but they cannot become eliminated. Thus, the outlet of STP, transports huge amount of substances, including PhACs, reaching the surface waters of the environments.

PhACs are classified in different effect classes. The relevant substances are analgesic, lipid lowering agents, anti-epileptic drugs, beta-blockers, hormone therapeutics and antibiotics. Due to former data, the following substances were mainly detected: beta-blockers (e.g. 1.54 µg/l Metoprolol), analgesic and anti-inflammatory compounds (e.g. 1.2 µg/l Ibuprofen); estrogens (e.g. Ethinylestradiol), gestagens (0.013 µg/l Levonorgestrel), as well as anti-epileptic drugs (e.g. 2.1 µg/l Carbamazepine). Therefore the pharmaceutical substances which were chosen to be analysed in the following study were Carbamazepine (CBZ), Ibuprofen (IBU), Levonorgestrel (LNG) and Metoprolol (MET). They are representing the most used treatment groups.

In comparison to the amount of available analytical data little is known about their accumulation and the biologically potency on non-target organisms in the aquatic environment. The current used detection methods are unspecific toxicity tests, which cannot show how specific biological or physiological effects of PhACs might be. Every biological effect, changes the homeostasis on organism, which could lead to a specific stress response.

The aim of the study was to assess whether PhACs might cause effects on the aquatic non-target organism, the free-floating macrophyt *Ceratophyllum demersum*. The non-target organisms were quantified as uptake and stress-biomarkers.

The following hypotheses were used for clarification:

- If and how much of the chosen PhACs are accumulated and metabolized in the macrophyte?
- Which effects have PhACs on chlorophylle pigments as a stress marker?
- If cell detoxification enzymes of *C. demersum* reacts in response to PhACs?

II.I Bioaccumulation and metabolites

Bioaccumulation is an interaction of passive uptake, accumulation and elimination. Furthermore, the biotransformation of PhACs is also involved in the process of detoxification:

- IBU and LNG were shown to be taken up by *C. demersum*.
- CBZ and MET also demonstrated incidences of bioaccumulation in *C. demersum*.
- The formation of first step metabolites (OH-IBU and *O*-desmethyl MET) were shown in the submerged macrophyte species *C. demersum*. The rates of metabolism of PhACs were generally significantly less than these of the uptake or bioaccumulation rate, resulting in significant PhACs bioaccumulation.
- PhACs affected the biotransformation and antioxidant related parameters after or during one-week exposure.

II.II Chlorophyll pigments

In *C. demersum*, chlorophyll pigments were not affected, yet a significant photosynthetic inhibition occurred, suggesting that the mechanism of action was mainly involving the interruption of the electron transport chain. The results indicated that PhACs (CBZ, IBU, LNG, and MET) can influence chlorophyll pigments, whereby *C. demersum* showed a higher sensitivity to MET than to the other PhACs tested:

- A reduction of total chlorophyll pigment (Chl total) was shown at the higher concentration of MET (4.1×10^{-3} mol/l)
- Only LNG was able to show incidences to inhibit the chlorophyll pigments at 1×10^{-8} mol/l LNG on the 4th day of exposure, but Chl total was recovered on day 7.

II.III Effects

Little is known on the distribution of toxins in plants, special macrophytes and signaling effects via reactive oxygen species. *C. demersum* exposed to PhACs showed diverse stress responses, which could lead to structural or biochemical changes. This provides a helpful tool to assess possible effects of PhACs on non-target organisms. Nonenzymatic antioxidants, like ROS, could be tested direct by or indirectly by over the regeneration of ascorbic acid or other components:

- The acute study demonstrated the occurrence of physiological changes in the oxidative stress response of *C. demersum* due to CBZ, IBU, LNG, and MET exposure.
- The non-target organisms showed different reactions such, as structural biochemical changes after exposure to PhACs.
- The enzyme mGST was not strongly affected during the exposure with the low concentrations of the PhACs used.
- Enzymes are localized on diverse places and could also have different functions, but are inhibited in the same way.

As the results show, further environmental relevant studies are needed to understand the mechanisms of accumulation, toxicity and antioxidative stress resistance of aquatic plants. The capability, that *C. demersum* could metabolize the pharmaceutically substances and also influence the enzyme activities, indicates that physiological biomarkers, as a supplement of chemical analyses, are useful for a more precise information. They specified the amounts of substances and are capable to develop a strategy to scope with the habitat and to reduce the effects of PhACs.

III. Zusammenfassung

Pharmazeutisch aktive Stoffe (PhACs) sind in allen anthropogen beeinflussten Oberflächengewässern ubiquitär, in physiologisch relevanten Mengen (> 1 µg/l), nachzuweisen. Gerade in Ballungsräumen werden Gewässer durch anthropogene Nutzung (durch das häusliche oder klinische Abwasser, veterinärmedizinische Verabreichung, etc…) maßgeblich beeinflusst. Mehr als 80 verschiedene PhACs und ihre Metaboliten konnten in Oberflächengewässern in limnischen, marinen Gewässern sowie in Gewässersedimenten nachgewiesen werden.

Humane oder im veterinärmedizinischen Bereich eingesetzte PhACs gelangen hauptsächlich durch die Kläranlage in die Umwelt. Die Konzentrationen von den therapeutisch verwendeten Medikamenten werden während der Verabreichung zwar reduziert, ebenso durch mikrobielle Degradation oder Absorption der Substanz am Klärschlamm, aber sie sind nicht vollständig eliminierbar. Fließgewässer werden als Vorfluter genutzt und transportieren trotz hoher Klärstufen eine Vielzahl potentiell schädlicher Substanzen. Innerhalb dieser Gewässer verzögert sich die Fließgeschwindigkeit, z.B. durch Mäandrierung, wodurch die aquatischen Organismen den Wirkstoffen länger ausgesetzt sind.

Es wurden weltweit verschiedene Substanzen in Flüssen detektiert, welche sich in verschiedene Wirkklassen unterscheiden lassen: Schmerzmittel, Lipidsenker, Antiepileptika, Beta-Blocker (*ß*-Blocker), Beta-Agonisten, Hormontherapeutika und Antibiotika. Für z.B. den *ß*-Blocker Metoprolol (MET) wurden Werte von 1.54 µg/l MET detektiert, für Analgetika und Anti-Inflammatorika (z.B. Ibuprofen) bis zu 1.2 µg/l IBU; der Wert für Estrogene (17ß-Estradiol) lag bei bis zu 0.013 µg/l, Gestagen (Levonorgestrel, LNG) und ebenso wurden Anti-Epileptika (z.B. Carbamazepine (CBZ)) bis zu 2.1 µg/l CBZ nachgewiesen.

Über den Verbleib der PhACs, ihre Akkumulation und ihre biologische Wirksamkeit in Makrophyten, ist bisher wenig bekannt. Weswegen diese Arbeit eine gesellschaftlich wichtige Relevanz aufweist. Die bisher angewandten Detektionsverfahren sind unspezifische Toxizitätstests, die außer Acht lassen, dass PhACs biologisch/physiologisch hochwirksame Stoffe sind. Jede biologische Wirkung verändert die Homöostase in einem Organismus, welcher sich als Stress äußern kann. Ein Ziel dieser Arbeit besteht darin, an einem aquatischen Nicht-Ziel-Organismus, dem freischwimmenden Makrophyten *Ceratophyllum demersum (C. demersum)*, erstmalig die biologische Wirksamkeit von PhACs darzustellen. Dies wird anhand der Aufnahme der Substanzen und an verschieden Stress-Biomarkern gemessen.

In der hier vorgestellten Arbeit wurden folgende Ergebnisse zur Klärung der Fragestellung herausgearbeitet:

- ob und wieviel von den jeweiligen PhACs akkumuliert und von den Makrophyten umgewandelt werden können;
- welche Auswirkungen PhACs auf Chlorophyll als Stressmarker haben;
- ob die Enzyme der Zelldetoxifikation auf die Anwesenheit von PhACs reagieren.

III.I Bioakkumulation und Metabolite

Die Bioakkumulation ist ein Zusammenspiel aus Aufnahme, Anreicherung und Ausscheidung, sowie auch die durch Biotransformation entstandenen Metabolite von pharmazeutisch aktiven Substanzen und deren Eliminierung:

- IBU und LNG weisen eine Aufnahme in dem Nicht-Ziel-Organismus *C. demersum* auf.
- Bei CBZ und MET hingegen kommt es zur Bioakkumulation in der Makrophyte.
- Die Formation von hydroxy-IBU (OH-IBU) und *O*-desmethyl MET (*O*-des MET) können in den freischwimmenden Makrophyte nachgewiesen werden. Die Rate der PhACs Metabolite ist generell wesentlich geringer und weist eine geringere Rate auf als das Ausgangssubstrat.
- in einem einwöchigen Versuch beeinflussen die verwendeten PhACs die Biotransformation und für Antioxidantien relevanten Parameter.

III.II Chlorophylle

Wenn auch in *C. dermersum* die Chlorophyll Pigmente nicht stark beeinflusst worden sind, kann es dennoch zu signifikante Photosynthese-Inhibition kommen, wodurch es zu Unterbrechungen im Elektronentransport kommen kann. CBZ, IBU, LNG und MET wiesen eine Tendenz zur Inhibition auf, wobei die größte Sensitivität bei MET auftrat:

- Die Tendenz zur Sensitivität gegenüber MET konnte in beiden Versuchskonzentrationen durch eine Tendenz zur Reduzierung des gesamten Chlorophyll-Pigments (Chl total) am siebten Tag (7d) nachgewiesen werden, wobei es bei der höheren Konzentration (4.1×10^{-3} mol/l MET) zu einer signifikanten Inhibierung der Pigmente kam.

- Nur bei LNG war es möglich eine Inhibition der Chlorophyll a-Pigmente bei der Konzentration 1 x 10^{-8} mol /l LNG am Tag 4 (4 d) der Exposition zu messen. Die Pigmente erholten sich aber zum Ende der einwöchigen Exposition wieder und wiesen keine weitere Inhibition auf.

III.III Enzyme

Wenig ist bekannt über die Verteilung von Toxinen in Pflanzen und den Signal-Wirkungen durch reaktive Sauerstoff Spezies. Der Nicht-Ziel-Organismus *C. demersum*, zeigt verschiedene Stressreaktionen, die von strukturellen bis zu biochemischen Veränderungen gehen können. Diese können dann wichtige Hinweise auf mögliche Effekte von PhACs auf Nicht-Ziel-Organismen liefern. Nicht enzymatische Antioxidantien, wie ROS, können direkt oder indirekt über die Regenerierung von Ascorbinsäure, oder andere Antioxidantien getestet werden:

- Die aktuelle Studie demonstriert Vorkommen von physikalischen Veränderungen während der oxidativen Antwort bei den freischwimmenden Makrophyten durch CBZ-, IBU-, LNG- und MET-Expositionen.
- Der Nicht-Ziel Organismus *C. demersum* zeigt verschiedene Reaktionen, wie strukturelle biochemische Veränderungen, wenn er den PhACs ausgesetzt ist.
- Das Enzym mGST wurde nicht beeinträchtigt bei der niedrigeren Konzentration der getesteten PhACs.
- Enzyme sind an verschiedenen Orten lokalisiert und besitzen dadurch auch unterschiedliche Funktionen, sind dann aber dennoch auf gleiche Weise inhibiert.

Die Ergebnisse zeigen, dass weitere umweltrelevante Studien erforderlich sind um die Mechanismen der Akkumulation, Toxizität und antioxidativen Stressresistenz von aquatischen Pflanzen zu verstehen. Die Fähigkeit, dass *C. demersum* die pharmazeutisch aktiven Substanzen metabolisieren kann und die Enzymaktivität beeinflusst, zeigt dass physiologische Biomarker als Ergänzung zur chemischen Analytik verwendet werden sollten. Dadurch können genauere Angaben gemachen werden, in welchem Maße die Substanzen sich auf die Umwelt auswirken, um aus diesen Ergebnissen eine Strategie zur Korrektur der Habitate zu entwickeln und den Einfluss der PhACs reduzieren zu können.

1. Introduction

Organic compounds, isolated from plants as well as synthesized by chemists, have been used as drugs for a long time. It has been widely observed that as Western populations age, the consumption of more and more drugs for a vast array of ailments increases, reaching hundreds of tons of pharmaceuticals per year within the European Union (Fent et al., 2006b; Quinn et al., 2009). Pharmaceutically active compounds (PhACs) may be totally and/or partially metabolized and excreted via urine or faecal matter finding their way into waste water treatment plants (WWTPs) (Scheytt et al., 2006). The removing or degradation of labile compounds is done in the conventional sewage treatment, generally the removing of drugs and by-products in municipal waste water streams is not effective (Chen et al., 2009; Metcalf et al., 2000; Ternes, 1998; Zenker et al., 2014). For this reason effluents from WWTPs are an important and continuous source of PhACs input into the aquatic environment (Brun et al., 2006; Hernando et al., 2004). Several of these compounds are among the most frequently prescribed drugs, where some of them are known to be environmentally persistent (Crane et al., 2006; Fent et al., 2006b). Numerous studies and reviews exist over the occurrence and quantities (mostly in the ng/l and µg/l range) in the effluent and receiving waters (Brun et al., 2006; Fent et al., 2006a; Gros et al., 2006; Heberer, 2002; Jones et al., 2002; Kolpin et al., 2002; Kümmerer, 2009). PhACs have been also detected in drinking water (Ternes, 1998). In comparison to the amount of available analytical data, little is known on their accumulation and ecotoxicological effects on non-target organisms in the aquatic environment.

1.1 Pharmaceutically active compounds (PhACs) in the environment

The reason why PhACs are interesting: they are developed by the intention to target particular metabolic and molecular pathways in order to elicit a specific biological response, which makes them to potentially harmful xenobiotics also for non-target organisms. Along the pathway, two effects contribute to enhanced risk: 1) some of these pharmaceuticals are not completely degraded over the course of a WWWTs (Carballa et al., 2004), 2) the metabolic and environmental degradation of the parental compounds produce a variety of compounds that may increase the concentration of waste water mixtures of biological harmful substances. PhACs always occur in combination with other environmental pollutants, e.g. industrial chemicals, pesticides, or personal care products (Escher et al., 2005). This taken together with their continuous release into the environment has generated concern as to their potential harmful environmental effects (Lishman et al., 2006). The physico–chemical behaviors often have the

same type as these naturally occurring in the cell, e.g. they are lipophilic in order to be able to pass through membranes, are persistent in order to overcome other substances and are inactivated before having a curing effect. Although the concentrations of individual pharmaceuticals found in receiving waters and effluents are relatively low, the combined concentrations of pharmaceuticals having similar modes of action (concentration addition) and those with differing modes of action (response addition) could prove ecotoxicologically to be significant (Brain et al., 2004). PhACs and other xenobiotics cannot be used for nutrition, but are nevertheless taken up, accumulated and in parts metabolized (Coleman and Blake-kajff, 1997). PhACs are classified in different effect classes. The relevant substances are analgesic, lipid lowering agents, anti–epileptic drugs, beta-blockers, hormone therapeutics and antibiotics (Tixier et al., 2003). Due to former data following substances were mainly detected: *β*-blockers (e.g. Metoprolol, 1.54 µg/l); (Scheurer et al., 2010; Ternes, 1998), analgestic and anti-inflammatory compounds (e.g. Ibuprofen); (Behera et al., 2012; Cleuvers, 2003; Weigel et al., 2004); contraceptions (e.g. Levonorgestrel); (Besse and Garric, 2009; de Alda et al., 2002; Kuch and Ballschmiter, 2000; Vulliet et al., 2007), as well as anti–epileptic drugs (e.g. Carbamazepine 2.1 µg/l); (Fent et al., 2006a; Ollers et al., 2001; Ternes, 1998; Vernouillet et al., 2010). Therefore the pharmaceutical substances which were chosen to be analysed in the following study (Carbamazepine (CBZ), Ibuprofen (IBU), Levonorgestrel (LNG) and Metoprolol (MET) (Tab. 1.1)) thus represent the most commonly PhACs used.

Tab. 1.1 Summary of used Pharmaceuticals

Pharmaceuticals / IUPAC	Treatment	Formula	Use
Carbamazepine / 5*H*-Dibenz[*b,f*]azepin-5-carbamide	anticonvulsant,	$C_{15}H_{12}N_2O$	Treatment of epilepsy, narcosis (membrane depolarisation)
Ibuprofen / [*RS*]-2-[4-[2-Methylpropyl] phenyl]propansäure	nantiinflammatory, analgesic and antipyretic	$C_{13}H_{18}O_2$	rheumatic disorders, pain and fever
Levonorgestrel / 17β-Hydroxy-18-methyl-19-nor-17α-pregn-4-en-20-yn-3-on	progestin;gestagenic Gonanes (ethinylated derivatives)	$C_{21}H_{28}O_2$	hormonal contraceptives, „mini pill“
Metoprolol / [*RS*]-1-[isopropylamino]-3-[4-2-methoxyethyl]phenoxy]propan-2-ol	β_1 receptor blocker	$C_{15}H_{25}NO_3$	blood pressure lowering agent

1.1.1 Carbamazepine (CBZ)

Carbamazepine (CBZ) (Fig. 1.1) is an anti-epileptic drug that enhances the lower barrier of spasm and therefore lower the hyperaesthesia of neural cells in the brain. Furthermore, it is used to treat bipolar depression. CBZ stabilizes the inactivated state of voltage-gated sodium channels; due to this blockage, the sodium influx decreases and the stimulus transfer is disturbed.

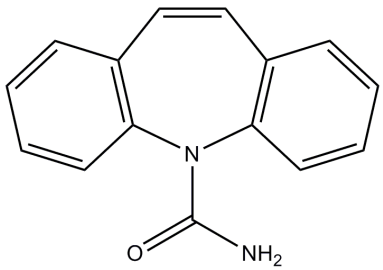


Fig. 1.1 Chemical structure of CBZ

CBZ has been found in surface waters in Canada at concentrations of up to 2.3 μg/l (Gagné et al., 2006), and in German wastewaters up to 6.3 μg/l (Ternes, 1998). Due to a sensitive method (Lajeunesse et al., 2009) which was developed for the determination of CBZ, the content could be detected in the algae *Pseudokirchneriella subcapitata* (Lajeunesse et al., 2009). Only few studies to date had used the bioactivity of algae to evaluate the eco-toxicological impact of CBZ (Ferrari et al., 2003; Vernouillet et al., 2010).

1.1.1 Ibuprofen (IBU)

Non-steroidal anti-inflammatory drugs (NSAID), such as Ibuprofen (IBU) (Fig1.2) possessing analgesic, antipyretic and anti-inflammatory properties, relieve symptoms of arthritis, rheumatic disorders, and fever when applied. NSAIDs reversibly inhibit by competing with its substrate, the cyclo-oxygenase (COX) activity which converts arachidonic acid to various prostaglandins and thromboxanes (FitzGerald and Patrono, 2001; Nelson and Cox, 2005). Buser and colleagues (Buser et al., 1999) described the excreted range of IBU to a significant degree of 70-80% of the therapeutic dose; apparent compound and/or in the form of derivates. The main metabolites of IBU are hydroxyl-ibuprofen (OH-IBU), carboxy-ibuprofen (CA-IBU), and carboxy-hydratropic acid (CA-HA) (Zwiener et al., 2002) (Fig. 1.3).

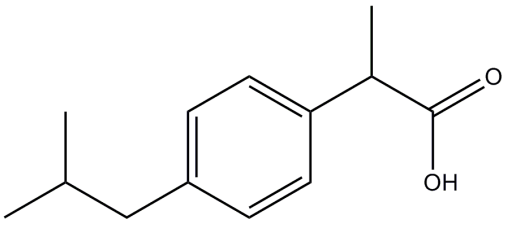


Fig. 1.2 chemical structure of IBU

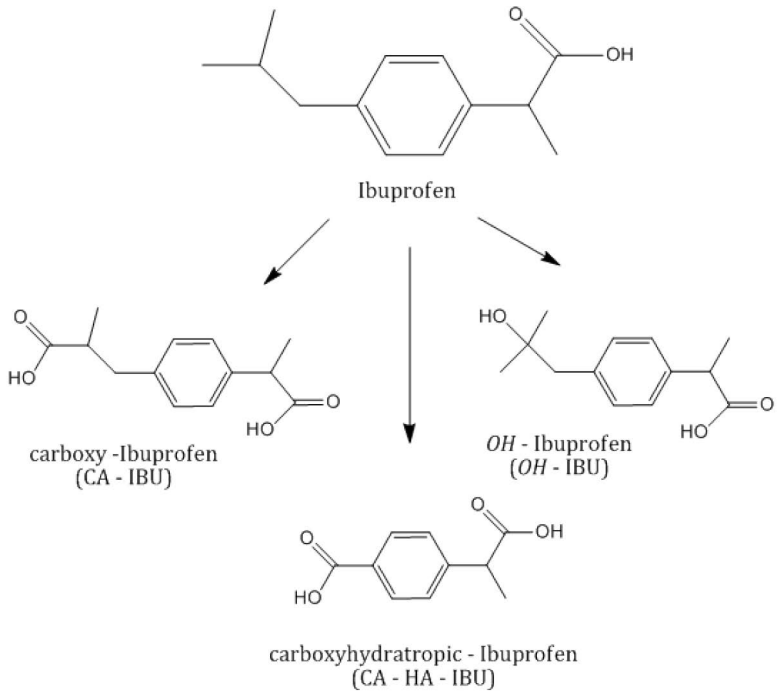


Fig. 1.3 Schematically structures of main metabolic pathways of IBU.

Daughton and Ternes (Daughton and Ternes, 1999) reported that the levels of IBU in surface waters can reach up to 0.53 µg/l. The modes of action and effects of NSAIDs and ß-blockers in non-target organisms, and also their route of uptake are not well understood (Cleuvers, 2003; Fent et al., 2006b; Owen et al., 2007). In a previous study it was shown that IBU and also CBZ are toxic to the green algae *Desmodesmus subspicatus* (Escher et al., 2005). Also Gonzáles-Naranjo and Boltes showed in their study that IBU is toxic to the green algae *Pseudokirchneriella subcapitata* and to the monocotyledonous *Sorghum bicolor* (González-Naranjo and Boltes, 2013).

1.1.2 Levonorgestrel (LNG)

Natural progesterone, also called gestagen, is an important regulator during oocyte maturation and pregnancy via progesterone receptor binding. Synthetic gestagens, such as Levonorgestrel (LNG) (Fig. 1.4) are able to inhibit ovulation and proliferation of the endometrium, and are thus applied as contraceptives. In urine 45% and in faeces about 32% of LNG and its metabolites are excreted, mostly as glucuronide conjugates.

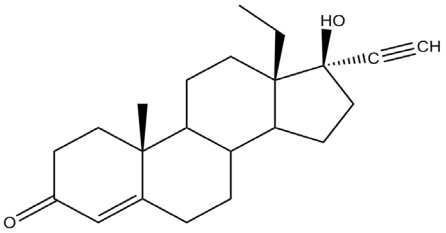


Fig. 1.4 Structure of LNG

The environmental relevance of synthetic gestagens, more specifically LNG, becomes apparent regarding the occurrence in surface waters at concentrations in the nanogram per liter range up to

30 ng/l (Viglino et al., 2008; Vulliet et al., 2007). LNG was classified as a highly active compound with a high risk to affect non-target organisms (Christen et al., 2010; Lorenz et al., 2011). A study with higher concentrations provoked the masculinization of females and the reduced the fertility of fish (Fick et al., 2010). Fish reproduction decreased when exposed to synthetic gestagens, even at concentration below 1ng/l (Zeilinger et al., 2009). To our knowledge there is no study available dealing with the effect of LNG on macrophytes or algae. The only known studies about steroid hormones in aquatic environments dealing specifically with macrophytes have so far focussed on bioactive estrogens (Shi et al., 2010). They showed an uptake of estrogens in algae (e. g. species of *Anabaena, Chlorococcus, Spirulina)* and duckweed (species of *Lemna*) (Shi et al., 2010).

1.1.3 Metoprolol (MET)

Metoprolol (MET) (Fig. 1.6) is a selective β1-adrenergic receptor antagonist belonging to the class of receptors critical to normal function of the sympathetic branch of the vertebrate autonomic nervous system. MET is used to treat angina and hypertension. It is also a potent membrane-stabilizing agent (Kim et al.,

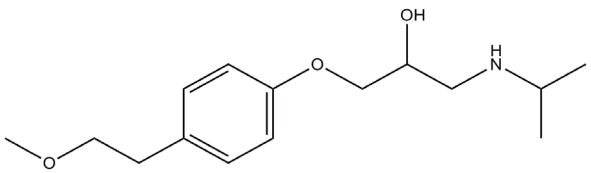


Fig. 1.6 The structure of MET

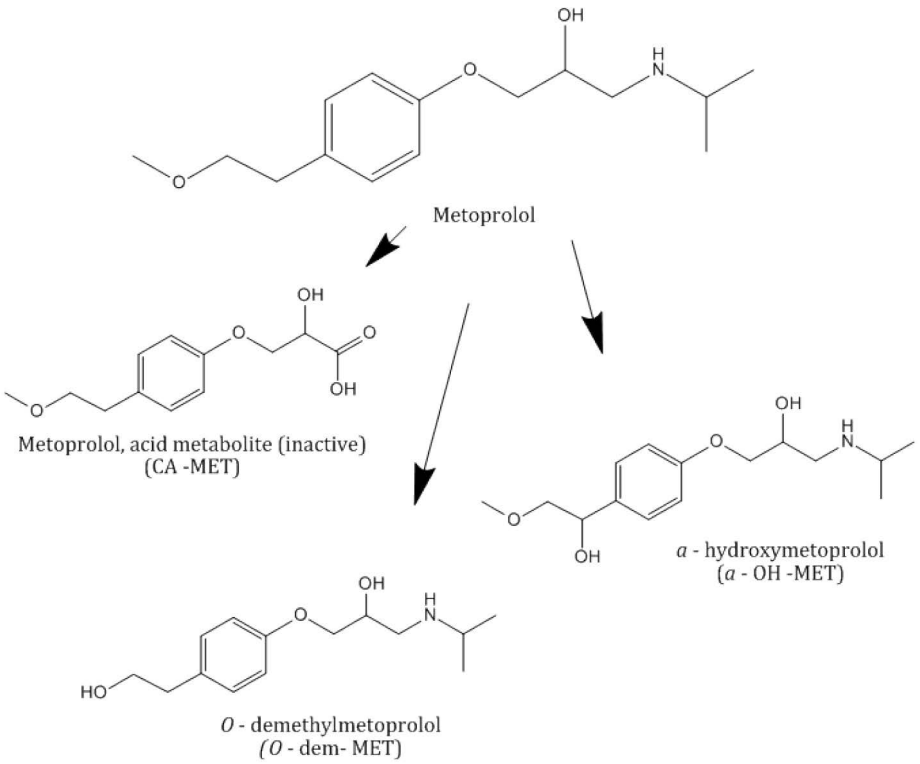


Fig. 1.5 Schematically chemical structures of the main metabolites of MET

2003).

The metabolites of MET are generated by aliphatic hydroxylation (α-hydroxymetoprolol, α-OHM), and O-dealcylation (O - dimethylmetoprolol, ODM) followed by oxidation (Borg et al., 1975) (Fig. 1.5).

This varies depending on the oxidation phenotype of MET (Boralli et al., 2009; Liang et al., 2010; Ma et al., 2007). It has been recognized that conjugation is an important pathway of many compounds in microorganisms and it was shown that MET could be transformed by *Cunninghamella* into different metabolites one of which was *O*-desmethyl MET (Ma et al., 2007).

MET has been detected in surface water in concentration of up to 2.2 μg/l (Scheurer et al., 2010). Cleuven (Cleuvers, 2005) showed with the green algae *Desmodesmus subspicatus* and the duckweed *Lemna minor* that beta-blockers have ecotoxicicological impacts. Also, in a previous study there has been shown that beta-blockers are toxic to the algae *Pseudokirchneriella subcapitata* (Radjenovic et al., 2011). Contardo-Jara (Contardo-Jara et al., 2010) was able to show physiological changes in the detoxification enzymes in freshwater mussels *Dreissena polymorpha* due to MET.

1.2 Bioaccumulation of PhACs in plants

Bioaccumulation in plants contributes to the circulation of PhACs in the food chain through active and passive absorption, and subsequent accumulation in their tissue. Plants are primary producers from the basis of the food web and are therefore an essential indicator for PhACs bioaccumulation. Species growing within PhACs tend to exhibit elevated levels of them in their tissues. Biotransformation can modify bioaccumulation of xenobiotics in an organism.

1.3 Biotransformation in plants

Plants always have to deal with natural organic toxicants to deter competitors and predators. Many synthesized chemicals are structurally similar to naturally occurring compounds, the same enzyme systems are involved in their detoxification. Most organic contaminants are lipophilic thus make them easy to be absorbed across lipid membranes. Plants have developed a wide range

of mechanisms to survive in a hostile environment and to overcome these peculiar constraints. One of the common strategies developed by plants is the use of chemicals for defensive purposes. Compounds get biotransformed, to become more water-soluble, and can be excrete more easily via transportation of micelles.

The biosynthesis of plants derived compounds is most commonly referred to as secondary metabolism. So far, approximately 50,000 chemicals have been identified from plants, and many more must exist in nature (St-Pierre and Luca De, 2000). The large diversity of compounds is achieved by an elaboration of basic structures, which are enzymatically modified by the change or introduction of chemical functional groups. The xenobiotic metabolism is a set of metabolic pathways that modify compounds. These pathways are a form of biotransformation and are present in all major groups of organisms. The detoxification of xenobiotics and their removal from the cells occur through a complex interplay between different phases. In some cases the free radical intermediates in xenobiotic metabolism can cause toxic effects associated with oxidative stress by themselves (Fig. 1.7).

Most drugs are metabolized by cytochromes of the P450 3A and 2C8 families (Stresser et al., 2000). The cytochrome P450 (CYP) enzymes have been implicated in a wide variety of processes such as pigment synthesis, defense mechanism against chemical removal and the synthesis of steroids (Nebert and Dalton, 2006). The cellular detoxification systems of plants dispose of xenobiotics by two sequential processes: the chemical and the compartment transformation.

The reactions responsible for the disposal of xenobiotics in plants consist of three types: phase I (activation) reaction, where this phase creates reactive sites in the xenobiotic by addition or exposure of functional groups, usually involving hydrolysis or oxidation. If the parental xenobiotic already possesses an appropriate functional group it can directly undergo phase II reaction.

Phase II reactions, are conjugations with sugar (e.g. glucuronic acid), amino acids (e.g. glycine) or other moieties (e.g. sulfonate). A further reaction is the GST activity (e.g. glutathione), which is implicated in the conjugation of hydroxylase (Anzenbacher and Anzenbacherova, 2001; Coleman and Blake-kajff, 1997; Nebbia et al., 2001; Pflugmacher and Sandermann, 1998). These reactions are catalyzed by a large group of broad specificity transferases, which in combination can metabolize almost any hydrophobic compound that contains nucleophilic or electrophilic groups. Phase II is responsible for linking metabolites produced during phase I to various water-soluble endogenous compounds, which are present in the cell at high concentrations (Foureman et al., 1989).

The phase II products are either nontoxic or less toxic than the parent compounds. The inactive conjugates formed in phase II, are exported from the cytosol by membrane–located transport proteins, which initiate phase III.

This is the compartmentalization and processing part of the detoxification system according to the so-called "Green liver concept" (Nimptsch et al., 2008; Pflugmacher and Sandermann, 1998). In phase III, the conjugated xenobiotics are processed further, before being recognized by efflux transporters and pumped out of cells. The subcellular compartments mediating phase III processes are the vacuole and the apoplast. This mechanism has been identified for many plants as a protection against cellular accumulation of environmental pollutants, as also excretion of non-metabolized substances via these transporters could be observed (Sandermann, 1992; Schröder et al., 2005; Van Eerd et al., 2003).

1.4 Reactive oxygen species and oxidative stress

Pathogens and wounding, or environmental stressors (e.g. temperature, drought or osmotic stress) have been shown to trigger the active production of reactive oxygen species (ROS) (Dröge, 2002; Kamara and Pflugmacher, 2007a; Peñuelas et al., 2005). ROS are continuously produced in plants as by-products of aerobic metabolism. The formation of ROS and the consequences in the cell is of great importance in the elucidation of essential questions for stress physiology. To balance redox metabolism and minimize ROS or reactive nitrogen species formation, cells operate a redox signaling network. The network senses environmentally induced redox imbalances and initiates compensatory responses either to readjust redox homeostasis or to repair oxidative damage (Dietz and Pfannschmidt, 2011; Livingstone, 1998).

ROS include oxygen ions, free radicals and peroxides, both inorganic and organic. They are generally very small, highly reactive molecules due to the presence of unpaired valence shell electrons. During the detoxification process superoxide will be catalyzed into oxygen and hydrogen peroxide. Hydrogen peroxide itself is toxic and needs to be further reduced.

The protonation of the formed superoxide anion radical and the consequent formation of perhydroperoxyl radical is one of the first steps of oxidative stress response.

$$H^+ + O_2^- \rightleftharpoons HO_2$$

A spontaneous dismutation of a pair of perhydroperoxyl radicals is followed to form hydrogen peroxide and, molecular oxygen.

$$*HO_2 + *HO_2^- \longrightarrow H_2O_2 + O_2$$

Superoxide anion and perhydroperoxyl radical undergo spontaneous dismutation to form hydrogen peroxide, molecular oxygen and hydroxyl anion.

$$*HO_2 + O_2^- + H_2O \rightarrow H_2O_2 + O_2 + OH$$

The enzyme catalyzed the two superoxide radicals by superoxide dismutases also yield hydrogen peroxide.

$$2O_2^- + 2H \rightarrow H_2O_2 + O_2$$

The reduced or activated derivates of molecular oxygen are highly reactive and toxic in both plants and animals.

1.5 Mechanism of defense against oxidative stress in plants

When susceptible plants are exposed to xenobiotics or chemical substances, there is an overproduction of ROS. A common property of all ROS types is that they can cause oxidative damage to cell compartments (Blokhina, 2003; Dat et al., 2000), by triggering a chain reaction (Fig. 1.7). The oxidative stress conditions lead to damage of important biomolecules such as nucleic acids, proteins, lipid pigments. The sugar and the base moieties of DNA are susceptible to oxidation, causing base degradation, single strand breakage, and cross-linking to protein (Imlay and Linn, 1988). The incurred molecule is electrically charged and therefore cannot pass through biological membranes.

Tab. 1.2 Subcellular locations of some antioxidant enzymes and molecules involved in plant defences.

Antioxidant Enzyme / Molecule	Subcellular location
Superoxide dismutase	Cytosol, mitochondrion, plastide, peroxisome
Peroxidases	Cytosol, cell wall-bound
Catalase	Cytosol, mitochondrion, peroxisome, glyoxysome
Gluthathione reductase	Cytosol, mitochondrion, plastid, chloroplast
Gluthathione S-transferase	Cytosol, microsomal
Ascorbate peroxidase	Plastid stroma and membranes
Reduced gluthathione (GSH)	Cytosol, mitochondrion, plastid,
Tocopherol (vitamin E)	Cell and plastid membranes
Ascorbate (vitamin C)	Cytosol, plastid, apoplast, vaucole

Source: (Hausladen and Alscher, 1993; Mittova et al., 2004; Scandalios, 2005)

1.6 Enzymatic defenses

Several physiological processes are sensitive to pollution (Bayne et al., 1979), and can hence be used as indicators of environmental stress. During normal cell respiration, approximately 0.1 – 0.2 % of the oxygen consumed by aerobic cells is converted into ROS, due to the uncompleted reduction of molecular oxygen in the mitochondrial electron transport chain (Fridovich, 2004). It is widely explained as a lack of balance caused by different factors; enhancing the production of free radicals ROS and causing damage known as related to oxidative stress. Enzymatic antioxidants are therefore a powerful defense mechanism in all cells (Tab. 1.2). The three major classes of antioxidant enzymes are the superoxide dismutase, the lipid-soluble radical scavengers like catalase (CAT), peroxidases (POD), and glutathione-S-transferase (GST) (Asada, 2006; Nimptsch and Pflugmacher, 2007; Polle, 2001) (Fig. 1.7). Lipids in membranes are one of the most common targets of the ROS, forming lipid peroxides. Quantification is one of the biochemical tools used to assess the damage provoked by oxidative stress. In addition, there are numerous specialized antioxidant enzymes reacting with detoxifying oxidant compounds.

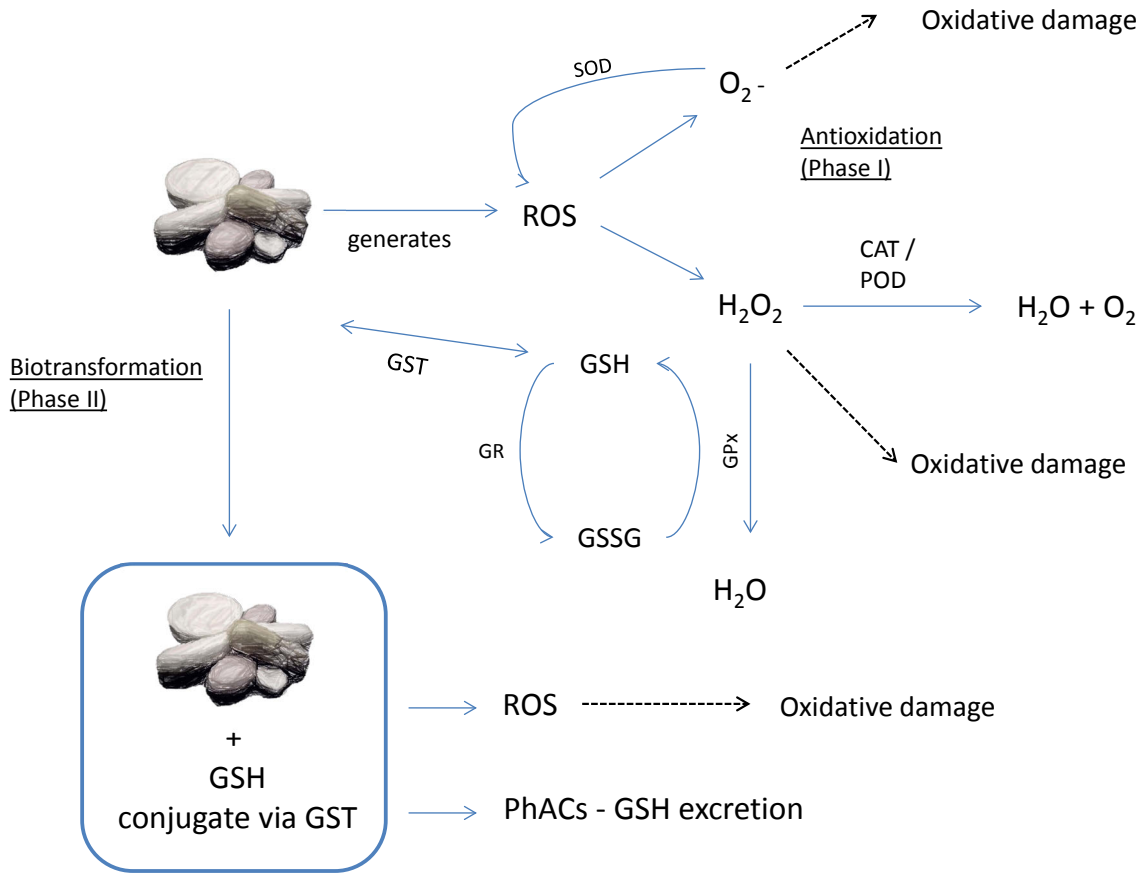


Fig. 1.7 Biotransformation and antioxidant schematic illustration (enzymes). Cell impact emerging by exposure to PhAC, can be detected within different enzymatic systems, mainly biotransformation and antioxidant defense system. CYP450: Cytochrome P450 m

The steady state ROS level in cells is regulated by antioxidant compounds such as thiols (glutathione), ascorbic acid (vitamin C), and carotenoids, α-tocopherol (vitamine E), flavonoids (in plants), amongst many others (Denisov and Afanas´ev, 2005). Superoxide dismutase is the first enzyme in a long enzyme order in antioxidative defense as is catalyzes the dismutation of two superoxide radicals to hydrogen peroxide to water and oxygen. The responses of these antioxidative enzyme and antioxidants to xenobiotic stress vary among plant species.

Superoxide dismutase (SOD) is an enzyme in antioxidative defence as it catalyzes the dismutation of two superoxide radicals to produce hydrogen peroxide and oxygen. The hydrogen peroxide is reduced by enzymes such as CAT, peroxidases, and ascorbate peroxidase (APx) the latter using ascorbate as electron donor (Asada, 1992). It therefore plays a pivotal role in the protection against oxidative damage and the adjustment processes of metabolic pathways. This could trigger the mitochondrial apoptotic signal through perturbation of mitochondrial function; with possible

leakage of cytochrome C, which is considered as secondary mechanism in addition to protein phosphatases inhibition.

1.6.1 Catalases (CAT:EC 1.11.1.6)

Catalases enhance the decomposition of hydrogen peroxide to water and oxygen, thereby protecting the cell from damaging effects of H_2O_2 accumulation (Fig. 1.7).

However, CAT are comparatively more efficient in dealing with relatively high concentrations of H_2O_2 because they exhibit a high K_m (in the millimolar range) for H_2O_2 (Fridovich, 2004, 1998); K_m being the concentration of substrate that leads to the half-maximum enzyme velocity. CAT activities are efficient H_2O_2–scavengers and can be photoinactivated in moderate light conditions to which plants are often adapted (Streb and Feierabend, 1996).

1.6.2 Peroxidases (POD:EC1.11.1.7)

Peroxidase enzymes are involved in detoxification processes. Their natural function is to prevent or minimize potential damages caused by hydrogen peroxide to plant cells and their constituents. Chloroplastic and cytosolic isozymes of ascorbate peroxidase have been known, and show some differences in enzymatic properties. Although hydrogen peroxide is formed during normal photosynthesis, the amount increases manifold when plants are exposed to stress conditions, leading to the dismutation of the superoxide radical and formation of H_2O_2. Castillo and colleouges (Castillo et al., 1987) used the activity of peroxidase enzymes as an indicator of pollution stress in the leaves of some forest trees and in the roots of *Pinus sylvestris*. POD enzyme catalyzes the reduction of hydrogen peroxide to water for detoxification (Fig. 1.7). Some peroxidase can also reduce alkyl hydroperoxidase to the corresponding alcohol (Fridovich, 1998).

1.6.3 Glutathione-S-transferases (GST:EC 2.5.1.18)

One of the important phase II enzymes are the glutathione-S-transferases (GST). These enzymes are involved in conjugating a variety of electrophilic metabolites, such as epoxides produced by CYP450. Glutathione (GSH) an endogenous tri-peptide, composed of the amino acids glutamate, cysteine, and glycine, is one of these conjugated metabolites (Hayes and Pulford, 1995). GST is found in cytosolic (also called soluble GST), microsomal (membrane bound, member of the membrane-associated proteins in eicosanoid and glutathione metabolism MAPEG family) and mitochondrial cell fractions (Hayes et al., 2005). GSTs hold a triple role in xenobiotic metabolism during oxidative stress. They are able to transport lipophilic toxicants to sites of phase I

biotransformation; conjugate xenobiotics to glutathione and form covalent bonds to activated metabolites, to get a water-soluble conjugate (Glatt, 2000; King et al., 2000).

Several herbicides and also natural toxins are detoxified by GST-catalysed gluthatione conjugation (Mitsou et al., 2006; Pflugmacher and Steinberg, 1997). Conjugation to glutathione takes place in the cytosol, but the accumulation of the conjugates in this compartment is potentially harmful. GST could respond differently to varying compound exposures. The conjugates may block the activity of GSTs (product inhibition), leading to a build-up of unconjugated electrophiles in the cell; or they may encounter cytosolic enzymes, which could form toxic metabolites. The algicide 1-chloro-2,4-dinitrobenzene (CDNB) is one of the most commonly used model substrates for the non-specific testing of the biotransformation profile of GST enzymes in plants and animals.

1.7 Photosynthesis and oxidative stress

An important anabolic process is the photosynthesis which takes place in the chloroplast (thylacoid membrane) of plants as well as of other autotrophic organisms such as algae and cyanobacteria. Photosynthesis is the process where radiant energy is converted into a chemically stable form (Strasser, 1997). It consists of successive redox reactions where light energy is absorbed by pigment complexes and transferred to the reaction centers of the photosystems (Fig. 1.8). The pathway of this energy transduction involves several physical and chemical mechanisms as well as many components. The strict control is to avoid the unintended electron transfer to other substrates with a positive relative redox potential. The absorbed energy of plant cells is transferred as excitation energy. The energy ultimately originates from the light-driven electron transfer from water to NADP+, which is performed by the photosynthetic apparatus of chloroplasts (Fig. 1.8). Photosynthetic efficiency is an indicator of a plant´s vitality or fitness in response to changes in its surrounding; and moreover it is sensitive to a wide variety of photosynthesis events, e.g. proton translocation, thylacoid stacking and unstacking ionic strength. In other cellular organelles such as peroxisomes and mitochondria metabolic homeostasis of ROS also plays a significant role (Foyer and Noctor, 2005; Scheibe et al., 2005). The dynamics of the redox state are important factors in the ROS signaling and ROS metabolic pathway in macrophytes (Baier and Dietz, 2005; Pflugmacher et al., 2006). This also generates highly reactive

oxygen species such as superoxide radicals, which are by-products of the reduction process of molecular oxygen (Fig. 1.8).

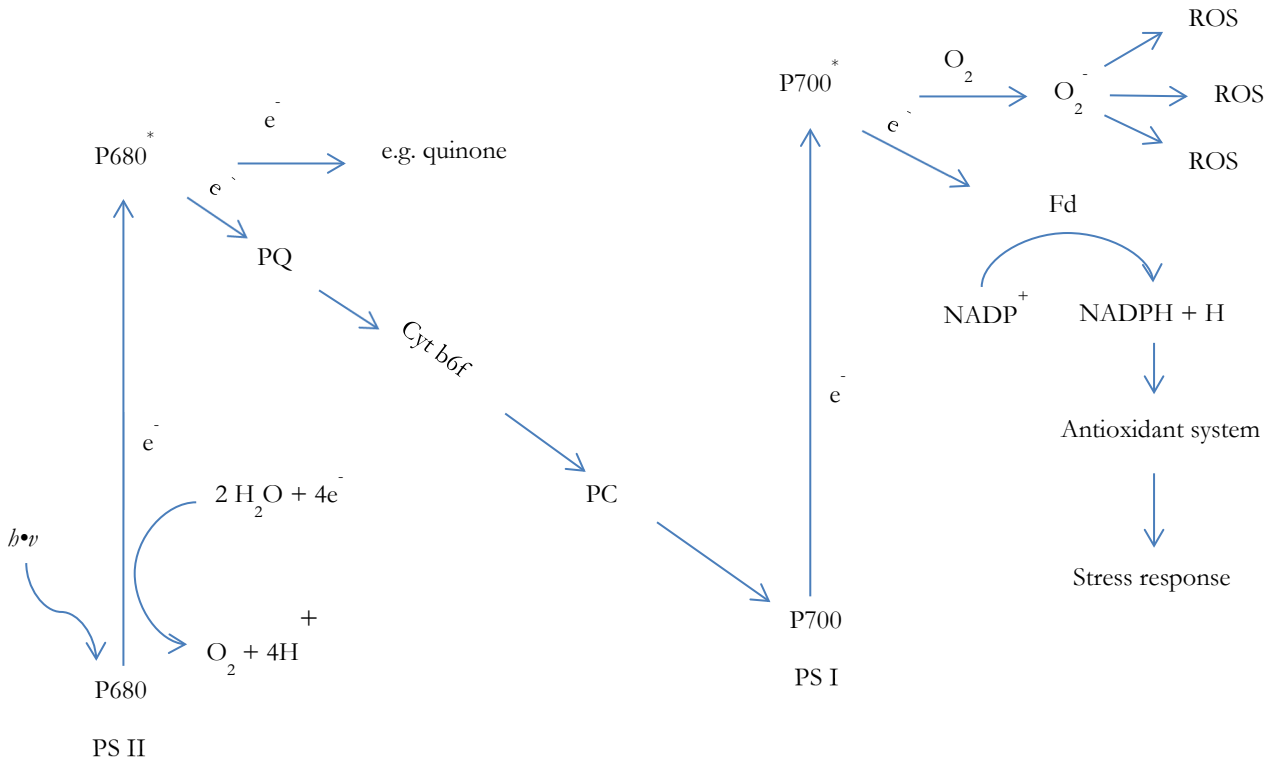


Fig. 1.8 Transport chain between photosythem II (PSII, P680) and photosystem I (PSI, P700) in a Z-schema. This diagram of energy, reveals the electron transport in a light dependent reaction of photosynthesis for plants and Cyanobacteria. Chlorophyll molecule in PS II becomes excited by radiation (*h•v*). Aromatic structures (e.g. quinones) in leaf litter extracts are hypothesised to interfere with the electron transfer from PS II to PS I, thereby slowing down H_2O photolysis and hence oxygen (O_2) and proton (H^+) release. The electrons from photolysis of water are transferred via plastoquinone molecules (PQ) and the cytochrome b6f (Cyt b6f) complex to PS I by a copper protein plastocyanin (PC). Excited chlorophyll molecule at PS I transmits electrons through the ferredoxin (FD) chain and eventually reduces NADP+ to NADPH. Electrons of PS I can also be transferred to oxygen, which results in the generation of a superoxide radical, a reactive oxygen species (modified from Dietz and Pfannschmidt, 2011; Kamara and Pflugmacher, 2007a).

Former studies have revealed that, when plants are influenced with xenobiotic compounds, there is usually an enhanced production of ROS (Esterhuizen et al., 2011). Bechthold and colleagues (Bechtold et al., 2005) have shown that stress conditions may influence a plant´s photosynthetic efficiency (Kamara and Pflugmacher, 2007b). Effects of environmental pollutants and toxicants on the photosynthetic activity of various algae species have been investigated (Kobbia et al., 2001). It is assumed that the fluorescence rise is rapid in changing the physiological state of the sample. This means that the structure and conformation of the sample remains constant and the variation in the fluorescent intensity are solely due to changes in the redox state of the reaction center complex of PSII. This is also reflected in the shape of the fast polyphasic fluorescence transient which has been shown to change upon modifications in various environmental

conditions, such as light intensity (Kruger et al., 1997; Srivastava and Strasser, 1997; Tsimilli-Michael et al., 1995), temperature (Guissé et al., 1995; Srivastava et al., 1997; Strasser, 1997), drought (Van Rensberg et al., 1996), or chemical influences (Quzounidou et al., 1997). Former studies pointed out that humic and (/) or humic-like substances could change the redox potential of components of the electron transport chain or of photosynthesis-coupled redox active compounds (thioredoxin or glutathione). They affected the electron transport efficiency (Kamara and Pflugmacher, 2007a; Pfannschmidt, 2003). A study with sorgoleone and sorgoleone-like compounds demonstrated the inhibition of the photosynthesis by disrupting the electron transfer between the plastoquinone A (QA) and the second plastoquinone B (QB) (Weir et al., 2004). A similar mechanism is suggested for the effects of natural organic matter on photosynthesis due to the quinones and aromatic compounds. The effects of the various factors are often indirect, and therefore not easily quantified and hard to be distinguished from one another. Fluorescence measurements have been successfully used to monitor and characterize a wide variety of photosynthetic events.

1.8 Aquatic macrophythes

Chemical contamination in aquatic environments is caused by the usage in human and veterinary medicine, but in fact organisms always have to deal with foreign compounds (xenobiotics, such as natural organic toxicants, to deter competitors and predators.

The responses of antioxidative enzymes and antioxidants to xenobiotic stress vary among plant species (Blokhina, 2003). Therefore, the distribution and abundance of aquatic plants is influenced by variations of environmental factors thus serving as gauges of ecological integrity. Macrophytes can cover large areas in aquatic environments and are known to accumulate pollutants by surface adsorption and/or absorption directly into their cells. Their sensitivity to short- and long-term changes in their surrounding makes them suitable indicators of environmental perturbations.

In a polluted aquatic ecosystem, macrophytes act as a bio-filter for xenobiotics (Doust et al., 1994; Ribeyre and Boudou, 1994; Vymazal and Kröpfelová, 2008). Plants are primary producers and are therefore an important indicator for bioaccumulation of PhACs and their effects (Shenker et al., 2011). Interactions between macrophytes and organic xenobiotics have been shown in accumulation and therefore physiological responses to these stressors (Yin et al., 2008).

Modulation of antioxidative enzyme activity has been used as a convenient model for the investigation of stress origination or various physiological responses from natural organic matter. This could be such as humic-like substances (Pflugmacher et al., 1999), or natural toxins (Esterhuizen-Londt et al., 2011; Pflugmacher et al., 2001) as well as other environmental pollutants (Nimptsch and Pflugmacher, 2005). Mohr and colleagues (Mohr et al., 2008) revealed that macrophytes reacted strongly to a single application of the highly effective biocide irgarol.

1.8.1 Submerged macrophyte: *Ceratophyllum demersum*

C*eratophyllum demersum* is a submerged freshwater perennial of worldwide distribution commonly found in ponds, marshes, and quiet streams. The stems are up to 7 m long, branched and brittle (Royal New Zealand Institute of Hortculture (RNZIH) 2005). *C. demersum* is rootless and free-floating, therefore avoiding the complications of soil-systems and root-shoot partitioning (Best and Visser, 1987). Furthermore the forked leaves of the plant and the thin cuticle on the plant surface area facilitate the uptake of nutrients as well as xenobiotics from water through its large surface.

C. demersum is ideal for laboratory tests and also useful as an oxygenator in closed equilibrated biological systems (Voeste et al., 2003). It is known that *C. demersum* is sensitive to stressors such as pure humic substances (Pflugmacher et al., 1999) and cadmium (Aravind and Prasad, 2005).

Detoxification mechanisms also exist in the macrophyte *C. demersum* (Pflugmacher and Steinberg, 1997; Pflugmacher et al., 1999). Additional oxidative stress and ROS may have implications on its growth and reproduction thus influencing its population dynamics and ultimately the ecosystem. Pflugmacher and colleagues found that the naturally occurring toxin microcystin has allelopathic effects on this aquatic macrophyte (Pflugmacher et al., 2001). Moreover, in a former study, Mishra and colleagues (Mishra et al., 2008) showed that *C. demersum* has the ability to accumulate toxic metals from water; but in general little is known about the effects of PhACs on this particular system.

1.9 Aim of the study

Pharmaceuticals are developed to affect specific biological pathways in target species. The biological effects of even low concentrations of these compounds on aquatic organisms, is so far porly reviewed (Fent et al., 2006a; Pomati et al., 2008).

This work has arisen as an attempt to unravel the mechanisms through which very potent and common pharmaceuticals affect one of the ecologically important non-target organisms, *C. demersum*. The biotransformation system powered by glutathione-S-transferase and the antioxidant enzymes, catalase as well as peroxidase, were chosen as relevant parameters in view of their participation in buffering PhACs toxicities and their potential participation in tolerance. In addition, and in order to connect the defense actions (biotransformation, antioxidants and redox system) within a broader biological-physiological framework, metabolic variables were assessed during the experiment to evaluate the extent of effects of PhACs.

Exposure to pharmaceuticals should lead to bioaccumulation in the macrophyte, and affect the cellular detoxification mechanisms. The toxicity of PhACs in *C. demersum* under laboratory conditions is to date not well studied. To our knowledge, the data base is limited to: *Lemna minor*, used to characterize the toxicity of MET and estrogens (Cleuvers, 2005; Shi et al., 2010); *Myriophyllum* spp., reacted strongly to a single application of Irgarol (Mohr et al., 2008); and the green algae *Desmodesmus subspicatus*, treated with IBU and CBZ (Escher et al., 2005).

The main objectives of this work were based on the following hypotheses:

- Using an organism with a moderate sensitivity as the non-target organism *C. demersum*, in active biomonitorin can indicate the uptake or bioaccumulation of PhACs.
  It was the aim to assess the quantities of PhACs via their concentrations in the macrophyte cells due to a steady-state exposure.
- Evaluation of detoxification processes as conjugation of the parental compounds in the macrophyte *C. demersum*.
  It was of interest to find out by using cellular PhAC metabolite concentrations, which are stored in the vacuole and which are stored in the cell membrane.
- The acute effect approached is applicable for the defense status of *C. demersum*.
  To evaluate if enzyme activities of biotransformation and antioxidation system is altered, due to exposure to different PhAC concentration and at different time points of the exposure.
- Possible differences in photosynthetic system and pigment content alterations might also be observable in the moderate sensitive non-target organism *C. demersum*.
  The effects of PhACs can be assessed through their effects on chlorophyll a and b content, as well as the total chlorophyll content.

2. Material and methods

2.1 Materials

2.1.1 List of equipment

Tab. 2.1 List of equipment and lab inventory used in this study

Equipment	Manufacturer	Model
Airflow controller	Waldner Electronics, Germany	Variolab w90
Autoclave 1	Varioklav, Germany	500
Autoclave 2	PBI international	Auto-Koch
Biological Safety Cabine	Heraeus, Germany	HS-12
Centrifuge 1	Eppendorf, Germany	Minispin
Centrifuge 2	Beckman Coulter, Germany	Optima Max
C18 column (CBZ)	Agilent, Germany	C18 Eclipse Plus
C18 column (MET)	Agilent, Germany	C18 Zorbax column XDB
Desalting columns	General Electric	Ilustra NAP-5
Desalting columns	Amersham Pharmacia	NAP -10
e-Ultra micro balance	Sartorious, Germany	Eclipse XDB-C8
Fixed angle-Rotor	Beckman Coulter, Germany	Type 70Ti
Freezer -80°C	Termo Life scientific, Inc	Forma scientific
guard column (CBZ)	Agilent, Germany	Supermicro S4
guard column (IBU)	Phenomenex, Germany	Phenyl-hexyl column
guard column (IBU)	Phenomenex, Germany	Luna Phenyl-hexyl column
Hemocytometer	Neolab, Germany	n/a
HPLC	Applied Biosystem, Germany	Agilent 1200 Series
Hot-plate	Neolab, Germany	Neoblockheiz duo
LC-MS/MS	Applied Biosystem, Germany	MS/MS: System 3200 Q Trap
Liquid nitrogen tank	Messergriesheim, Germany	Apollo
Microplate reader 1	Tecan, Switzerland	Infinity M200
Microplate reader 2	Tecan, Switzerland	Spectraflour plus
Microplate shaker	Heidolph, Germany	Titramax 100
Microscope	Leica-Leitz, Germany	Laborlux 5

Equipment	Manufacturer	Model
Oven 1	Heraeus, Germany	B6030
Oven 2	WTC Binder, Germany	n/a
PFP column (LNG)	Varian, Germany	Pursuit
pH meter	Inolab, USA	WTW Series 720
Photometer	Flowspek, Swiss	Uvikon^{TM} XL
pre-column (MET)	Agilent, Germany	Eclipse Zorbox XDB
Precision Balance	Kern & Sohn GmbH, Germany	EW420-3NM
Precision balance (electric)	Sartorius, Germany	BP 310 S
Shaker / temp control	Eppendorf, Germany	Thermomixer comfort
Stereo microscope	Olympus, Japan	SZ-60
Stirrer: Multi	Variomag, USA	Poly 15
Stirrer: Single / hot plate	Ika, Germany	Ret Control Visc Ikamag
Ultracentrifuge	Beckmann Coulter, USA	Optima L-90 Ultracentrifuge
Vacuum filter:	Sartorious, Germany	n/a
Water purification	Millipore, USA	Milli-Q synthesis

2.1.1.1 Chemicals

Carbamazepine (CBZ) ≥98 %, Ibuprofen sodium salt (IBU) ≥98%, D(−)-Norgestrel (Levonorgestrel) (LNG) ≥99 % and (±)-Metoprolol (+)-tartrate salt (MET) ≥98 % obtained from Sigma-Aldrich (Germany). If not further indicated, all pharmaceuticals were dissolved in ion exchange and filtered water (Milli-Q-grade) and used for exposure stock and LC-MS/MS reference preparation. For the exposure stock solutions and the LC-MS/MS references solutions MET and IBU were dissolved in water (LC-MS/MS-Grade). LNG and CBZ were dissolved in ethanol (LC-MS/MS-Grade). The concentration of ethanol in the exposure medium has to be kept well below 2 % (v/v) caused by toxic effect (Escher et al., 2005).

Tab. 2.2 List of Chemicals used in this study

Chemical	Molecular formula	Company
Advanced solution for protein determination	ADV1	Cytoskeleton, Inc
Acetonitrile	CH_3CN	Rathburn, UK
acetic acid	HCOOH	Sigma-Aldrich, Germany
Ammonium chloride	NH_4Cl	Merck, Germany
Ammonium molybdate	$(NH_4)_2MoO_4$	Merck , Germany

Chemical	Molecular formula	Company
Ammonium sulfate	$(NH_4)_2SO_4$	Sigma-Aldrich, Germany
Ammonium salt	CH_5NO_2	Sigma-Aldrich, Germany
Anthrone	$C_{14}H_{10}O$	Fluka, Germany
Boric acid	H_3BO_3	Merck, Germany
Bovine Serum Albumin		Sigma-Aldrich, Germany
Cadmium nitrate	$Cd(NO_3)_2$	Merck, Germany
Calcium chloride	$CaCl_2$	Sigma-Aldrich, Germany
Calcium nitrate	$Ca(NO_3)_2$	Merck, Germany
Carbamazepine (CBZ)	$C_{15}H_{12}N_2O$	Sigma-Aldrich, Germany
Chloroform	$CHCl_3$	Roth, Germany
Chromium nitrate	$Cr(NO_3)_2$	Merck, Germany
Copper sulfate	$CuSO_4$	Merck, Germany
DEPC - water		Roth, Germany
Dimethyl sulphoxide (DMSO)		Sigma-Aldrich, Germany
Di-potassium hydrogen phosphate	K_2HPO_4	Sigma-Aldrich, Germany
Di-sodium hydrogen phosphate	Na_2HPO_4	Sigma-Aldrich, Germany
Dithioerylthritol (DTE)		Sigma-Aldrich, Germany
5,5-Dithio-2-nitrobenzoic acid (DTNB)		Merck, Germany
Ethanol (absolute)	C_2H_5OH	Roth, Germany
Ethylene diamine tetraacetic acid (EDTA)		Sigma-Aldrich, Germany
L- Glutathione (GSH)		Sigma-Aldrich, Germany
Glucose		Sigma-Aldrich, Germany
Guaiacol		Sigma-Aldrich, Germany
Glycerol		Sigma-Aldrich, Germany
Hydrogen chloride	HCl	Merck, Germany
Hydrogen peroxide	H_2O_2	Aldrich, USA
Ibuprofen		Sigma-Aldrich, Germany
Liquid nitrogen		Messner, Germany
Levonorgestrel		Sigma-Aldrich, Germany
Magnesium sulfate	$MgSO_4$	Merck, Germany
Manganese sulfate	$MnSO_4$	Merck , Germany
Methanol	MeOH	Carl Roth, Germany
Metoprolol-tartrate salt		Sigma-Aldrich, Germany
Nicotinamide adenine dinucleotide	NADH	Sigma-Aldrich, Germany
N, N-dimethylformamide		Sigma-Aldrich, Germany
Potassium hydrogen carbonate	$KHCO_3$	Sigma -Aldrich, Germany
Potassium chloride	KCl	Roth, Germany

Chemical	Molecular formula	Company
Sea salt		Sigma-Aldrich, Germany
Sodium hydrogencarbonate	$NaHCO_3$ $NaHCO^3$	Merck, Germany
Sodium chloride	NaCl	
Sodium dihydrogen phosphate	NaH_2PO_4	Sigma-Aldrich, Germany
Sulfuric acid	H_2SO_4	Merck, Germany
Thiobarbituric acid (TBA)		Sigma-Aldrich, Germany
Trichloroacetic acid (TCA)		Fluka, Germany
Tris (hydroxymethyl) aminomethane	$(HOCH_2)_3CNH_2$	Roth, Germany
1-chloro-2,4-dinitrobenzen	CDNB	n/a
1,4-Dithioerythritol (DTE)		Sigma-Aldrich, Germany

2.1.2 Software

Tab. 2.3 List of software used in this study

Software	Company	Version
Adobe® Photoshop®	Adobe, USA	Version 8.0
Chem DrawULtra	PerkinElmer Inc.	Ver. 13.0
Microsoft Office	Microsoft, USA	Version XP
Mendeley	Mendeley Ltd.	Version 1.10.3
Origin	Origin LAB	OriginPro8G
PSAW software	StatSoft, Inc	PSAW Version 17
Ref Manger	Thomas Reuters	Ver. 5.0
Statistik analyse	StatSoft, Inc	2000
SPSS software	SPSS ink	SPSS ink Chicago IL USA

2.1.3 Plant material

Ceratophyllum demersum L. (Coontail)

Fig. 2.1 *Ceratophyllum demersum* L., Coontail, common Hornwort in the Ceratophyllaceae family (illustration source: imageenvision.com)

Tab. 2.4 Taxonomic hierarchy

Taxonomic hierarchy	
Kingdom	*Plantae*
Phylum	*Magnoliophyta*
Class	*Magnolionsida*
Order	*Nymphaeales*
Family	*Ceratophyllaceae*
Genus	*Ceratophyllum*
Species	*C. demersum* L.

2.2 Methods

2.2.1 Macrophyte cultivation and experimental setup

C. demersum were first rinsed with aquarium water to remove adhering debris in medium containing de-ionized water, calciumchloride ($CaCl^2$) (0.2g/l), sodium hydrocarbonate ($NaHCO^3$) (0.103g/l) and sea salt (0.1g/l) in a 100 l tank two weeks prior to exposure experiments for acclimatization (Pflugmacher et al., 2003). Supplementary light was provided by day light lamps with an irradiance of 100 µE m^2 s^{-1} at a light : dark cycle of 14 : 10 h. Temperature was held at 20 ± 1 °C.

For the bioaccumulation, metabolite, and chlorophyll analyses, 5 g fresh weight (FW) of submerged free-floated macrophytes *C. demersum* were used, and exposed for 1, 4 and 7 days (d), respectively in 500 ml beaker. The exposures for the enzyme analysis were set up with 10 g FW of *C. demersum* for 1 and 7 d in a 600 ml beaker. These exposures were done separately and with different concentrations of CBZ, IBU, LNG and MET (Tab. 1.1) in a static experimental set-up. All samples were taken randomly of each exposure concentration. After exposure, the plant material was washed three times in dest. water (H_2O) and methanol (MeOH) to remove residuals of PhACs from the surface. Further, the plant material was stored at −80° C until the tissue extraction. Control samples were taken at each time point.

2.2.2 Bioaccumulation and metabolite extraction and analyses of PhACs

2.2.2.1 Bioaccumulation and metabolite extraction of PhACs

The frozen tissue was ground, weighed (3 g) and homogenized in 20 ml dichloromethane : methanol : water (1 : 2 : 0.8) according to Bligh and Dyer (Bligh and Dyer, 1959), followed by the addition of 5 ml dichloromethane. The samples were kept dark at 4° C for 24 h, and after incubation the samples were centrifuged at 1,500 xg for 5 min. The organic phases were collected and evaporated to dryness at 25° C overnight. The samples from MET and IBU were dissolved in 250 µl 0.1 % formic acid, LNG and CBZ samples were suspended in 250 µl methanol (LC-MS/MS-Grade, Roth), followed by measurement with LC-MS/MS .

2.2.2.2 Bioaccumulation and metabolite analyses of PhACs via mass spectrometry

The same LC-MS/MS system was used for all water and tissue analyses. The system consisted of HPLC (Agilent 1200 Series) combined with the MS/MS system, 3200 Q Trap from Applied

Biosystems (Darmstadt). The pharmaceutical specific MS/MS conditions, ionization and fragmentation settings, were optimized by direct injection of standard solutions to the MS/MS system. The same injection volume of 25 µl per sample was used in all cases. The tandem MS/MS detections were carried out with Applied Biosystems 3200 Q Trap (Darmstadt, Germany). The treatments CBZ, LNG, MET and also the metabolites were performed in the positive MRM mode, and for each with specific mass - transitions. For IBU the mass spectrometric measurement was performed in the negative MRM mode. All data were analysed by using Analyst 1.4.2 software.

Carbamazepine

The medium concentrations for CBZ in the experiment were set at 0.8 x 10^{-5} mol/l (2.03 ± 0.11 mg/l CBZ) and 0.9 x 10^{-4} mol/l (21.36 ± 3.23 mg/l). The chromatographic separation of CBZ was performed on a C18 Eclipse Plus (RP 18, 4.6 * 50 mm, 5 µm) proceeded by a guard column Eclipse XDB- C8 (C8, 2.1 * 12.5, 5 µm) both from Agilent. The column oven temperature was maintained at 20° C. The flow was set to 400 µl/min within a binary composition of a mobile phase consisting of water (A) and methanol (B); each charged with 10 mM formic acid ammonium salt (CH_5NO_2) within 6 min. The gradient conditions were started with 100 % of A and stopped after 10 min with 100 % of B. The CBZ retention time was 2 min.

The measurements were performed with the mass-transitions of (m/z) 237.2 → 192.0 (Breton et al., 2005). The method validation was achieved over a linear range from 0.5 - 250 µg/l. Limit of detection (LOD) was 0.2 µg/l CBZ. The metabolites of CBZ were also measured under the same conditions as the CBZ standard (Breton et al., 2005). CBZ was measured in the medium and in all CBZ exposed macrophytes.

Ibuprofen

In the 7 d lasting experiment, 2.5 x 10^{-3} and 2.23 x 10^{-5} mol/l of IBU was used. The applied concentration in the medium of 2.5 x 10^{-3} mol/l IBU was 672.00 ± 387.50 mg/l, and in the second concentration (2.23 x 10^{-5} mol/l IBU) it was measured at 2.53 ± 0.10 mg/l IBU.

For IBU a LC-MS/MS equipped with a guard phenyl-hexyl column (4 mm * 2 mm, 5 µm pore size) combined with a Luna phenyl-hexyl column (50 mm * 3 mm, 5 µm pore size) both from Phenomenex were used for the separation. The column temperature was set at 65°C. The mobile

phase consists of methanol + 10 mM tributylamine (TrBA) + 0.5 % acetic acid (HCOOH), the flow rate was set to 150 µl/min under isocratic conditions for 2 min.

The most abundant ion was selected and the mass-spectrometer was set to monitor the transitions of the precursor to the product ion (m/z) 205.1 → 161.2 (Jones et al., 2012). The method was calibration made over a linear range from 10-100 µg/l (R^2 = 0.999). LOD was 3 µg/l IBU.

Levonorgestrel

Treatment concentration of LNG was at the max 1.06 x 10^{-7} (33.10 ± 3.06 µg/l LNG), and at the min 0.97 x 10^{-8} mol/l (3.03 ± 0.15 µg/l LNG) which are environmentally relevant concentrations.

The following LNG analysis was performed using a HPLC Agilent 1200 Series with a Pursuit PFP column (50 * 2.0 mm, 5 µm), from Varian (Germany). The column temperature was 30°C for LNG, with a flow rate of 500 µl/min.

The gradient program began with a hold for 2 min at 100 % of a mixture of solvent B (H2O + 0.1% HCOOH), followed by 65 % solvent A (Acetonitrile + 0.1 % HCOOH) for 5.5 min, and a final return to 100 % solvent B and 0 % solvent A for 2 min. All measurements were made with the mass-transitions of (*m/z*) 313.1 → 245.1 (Contardo-Jara et al., 2011). A linear calibration was made from 1-100 µg/l, and a detection limit of 0.5 µg/l. Between 0.5 and 50 µg/l the linear calibration was determined (R^2 = 0.99).

Metoprolol

The used treatment concentration of MET was at the min 1.8 x 10^{-5} mol/l (4.81 ± 0.340 mg/l) and at the max 4.1 x 10^{-3} mol/l (1091.33 ± 327.60 mg/l MET). For the MET analysis the HPLC was equipped with a pre-column (Eclipse XDB 12.5 x 2.1 mm, 5 µm) and a C_{18} column from Zorbax XDB (50 x 2.1 mm, 5 µm). The column temperature was kept constant at 30°C. The gradient program began with a hold for 12 min at 97 % of a binary mixture of solvents A (H_2O + 1 % MeOH + 0.1 % HCOOH) and 3 % solvent B (MeOH + 0.5 % HCOOH), followed by 72 % solvent A and 28 % solvent B for 6 min, 47 % solvent A and 53 % solvent B for 2 min, and a final return to 97 % solvent A and 3 % solvent B for 10 min, performed at a flow rate of 200 µg/l. The MET retention time was 15 – 16 min.

All mass spectrometric measurements were made with the mass-transitions of (m/z) 286.3 → 133.1 and (m/z) 286.3 → 159 (Hernando et al., 2004). The LC-MS/MS system was validated using a linear range of dilutions of a MET standard determined from 0.5 - 50 µl/l.

Metabolites

The metabolites of IBU, hydroxyl-IBU (*OH*-IBU), were also measured in negative MRM mode during conditions like the IBU standard. OH-IBU had a precursor ion at 221.0 m/z and the product ion was 177.0 m/z obtained by the LC-MS/MS (Grillo and Hua, 2008) (Tab. 2.5).

MET metabolite (*O*-desmethyl-MET) was detected in positive MRM mode using the same conditions like the MET standard. The precursor ion was at 254.2 m/z and the production was 116.2 m/z obtained by LC-MS/MS analysis (Liang et al., 2010).

Carbamazepine–epoxide, the metabolite of CBZ, was also measured in positive MRM mode, the precursor ion was at 253.3 m/z and the production was obtained at 180.3 m/z, within the same conditions as the CBZ standard. No biotransformed substances could be detected for CBZ and LNG.

Tab. 2.5 Summary of metabolites precursor ions and their product ions which were used for measurement

Parent component	Metabolites of parent compounds	Precursor ion *(m/z)*	Product ion *(m/z)*	Paper; published year
CBZ	Carbamazepine-epoxid	253.3	180.3	(Breton et al., 2005)
IBU	*OH*-IBU	221.0	177.0	(Grillo and Hua, 2008)
MET	*O-desmethyl* MET	254.2	116.2	(Liang et al., 2010)

2.2.2.3 Bioconcentrationfactor (BCF)

The bioaccumulation of PhACs in *C. demersum* was calculated after the duration of the exposure. The bioconcentration factor (BCF) after 7 days based on the fresh weight was determined by:

$$BCF = \frac{C_b}{C_w}$$

Where C_b is the concentration of the organisms (mg/kg) and C_w is the concentration of water (mg /l). Therefore the factor could be estimated and it is related to the balance or saturation range of the substance, and there uptake and elimination. The factor is dimensionless:

$$BCF = \frac{C_1}{C_2}$$

C_1 is the uptake - velocity constant (l/kg or l/h); C_2 is the elimination-velocity constant (l/kg or l/h).

2.2.3 Chlorophyll extraction and determination

Chlorophyll a (Chl a), b (Chl b) and chlorophyll total (Chl total) content were analysed with 1 ml *N, N*-dimethylformamide using 0.25 g of plant material. Per concentration and each time point, five independent replicates (n = 5) were sampled for all pigment analyses. The samples were kept in darkness at 4° C and for 3 d, until the plant material was completely colourless, to allow for pigment extraction. The samples were centrifuged at 14,000 xg for 2 min and the absorption of the supernatant at optical density (OD) of 647 nm and 664.5 nm was measured against *N, N*-dimethylformamide. Handling and measurement of pigment samples was carried out in semi-darkness in order to avoid the influence of light on the pigments. Chl a, chl b and chl total contents were calculated and expressed as milligrams per gram fresh weight based on the extinction coefficients according to Inskeep and Bloom (Inskeep and Bloom, 1985).

Chl a = $(12.7 \times ABS_{664.5}) - (2.79 \times ABS_{647})$

Chl b = $(20.7 \times ABS_{667}) - (4.62 \times ABS_{644.5})$

Chl total= $(17.9 \times ABS_{647}) + (8.08 \times ABS_{644.5})$

2.2.4 Multi-enzyme extraction

Protein extraction of the enzymes catalase, glutathione-S-transferase (soluble and microsomal) and guaiacol peroxidase of *C. demersum* cell was performed as described in Pflugmacher and colleagues (Pflugmacher et al., 2001). Each of the frozen 10 g FW samples were individually

ground to a fine powder using nitrogen, and homogenized using a glass potter in ice cooled sodium phosphate buffer (0.1 M pH 6.5) containing 20 % glycerol, 1 mM ethylenediaminetetraaceti acid (EDTA), and 1.4 mM dithioerylthritol (DTE). Cell debris was removed by centrifugation of the slurry at 10,000 xg for 10 min, followed by a second centrifuge step at 5,000 xg for 5 min. The supernatant from the two centrifugations was then centrifuged to separate microsomal and cytosolic fractions at 40,000 xg for 60 min. The resulting pellets, containing membrane fractions and defined as the microsomes, were resuspended in 1 ml of 20 mM sodium-phosphate buffer (pH 7.0) containing 20% glycerol and homogenized using glass potter. The samples were desalted by gel filtration on NAP-10 columns eluted in 1.5 ml sodium buffer (50 mM). The extracts were immediately snap-frozen with liquid nitrogen and stored at -80°C until subsequent analysis.

The supernatant was precipitated with solid ammonium sulphate in two steps, 0 – 35 % and 35 - 80 % saturation followed by centrifugation at 20,000 xg for 20 min and at 30,000 xg for 30°min, respectively. The precipitate from the second precipitation which contains soluble protein-pellets was resuspended in 0.5 ml solution phosphate buffer (20 mM, pH 7.0) and desalted by gel filtration using NAP-5 columns (Amersham Pharmacia, Germany) which had been equilibrated with cytosolic buffer (Na_3PO_4 20 mM, pH 7). The eluted and now purified cytosolic (soluble) protein fraction (1 ml) was also collected in 1.5 ml Eppendorf tubes, immediately snap-frozen in liquid N_2, and stored at - 80°C until enzyme analysis could be performed.

2.2.5 Determination of protein concentration

The protein content of each sample was determined by the method of Bradford (Bradford, 1976) using the Bradford protein dye reagent. However, when protein contents in samples were below detection limits for Bradford reagent, a more sensitive "Advanced Protein Assay (ADV01)" using "Advance Solution" from cytoskeleton was employed. The disadvantage of the advance protein method is that a relatively large amount (800 µl compared to 25 µl per cuvette for the Bradford reagent) of sample material was required, but this is compensated for by the very high sensitivity of the reagent, making it possible to dilute samples up to a factor of 50 times. The total protein content was determined by a change in maximum absorbance from a wavelength of 465 nm to 595 nm of Coomassie Brilliant Blue G - 250 which is measured spectrophotometrically. Bovine

serum albumin (BSA 98 %) was used as a standard for calibration in both assay methods. A new calibration curve was prepared for every experiment. The protein assay mixture was composed of:

Bradford ($\lambda_{595\ nm}$)

Reagent	Volume (µl)
Bradford reagent	1225
Sample/H_2O (blank ref.)	25

Advanced ($\lambda_{595\ nm}$)

Reagent	Volume (µl)
Adv01 reagent	100
Sample/H_2O (blank ref.)	800

2.2.5.1 Biochemical assays

Catalase activity assay

Catalase (CAT: EC 1.11.1.6) activity was assayed by measuring the initial rate of disappearance of hydrogen peroxide (H_2O_2) at 240 nm on a spectrophotometer. The activity was calculated using the extinction coefficient (ε = 0.0361 l/mmol x cm) of H_2O_2 (40 mM/cm at 240 nm) (Baudhuin et al., 1964; Claiborne et al., 1986).

Catalase assay

Reagent	Volume (µl)
NA_3PO_4-buffer (50 M, pH 7.0)	1250

Sample/H_2O (blank ref.)	100

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

$$(\downarrow Abs\ = 240\ nm)$$

Peroxidase activity assay

Peroxidase (POD; EC 1.11.1) specific activity in the soluble fraction was measured spectrophotometrically using guaiacol as substrate according to Drotar (Bergmeyer, n.d.; Drotar et al., 1985). The principle of this assay is that guaiacol is oxidized in the presence of hydrogen peroxide and polymerizes to octahydrotetraguaiacol, followed by a change in the colour and absorbance which is monitored at a wavelength of 436 nm (extinction coefficient ε = 25.5 l/mmol x cm). Measurements were made in triplicates along with a blank reference over a period of 5 minutes duration. Samples were diluted fifty times in cytosolic buffer (NaP 20 mM, pH 7.0). The assay mixture was made of the following:

Peroxidase assay

Reagent	Volume (µl)
NA_3PO_4-buffer (0.1 M, pH 5.0)	860
Guaiacol solution (DMSO)	40
H_2O_2 (200mM)	40
Sample/H_2O (blank ref.)	40

$$Guaiacol + 2H_2O_2 \xrightarrow{\text{POD (pH 5.0)}} Tetraguaiacol + 2H_2O + O_2$$

$$(\downarrow Abs = 436\ nm)$$

Glutathione-S-transferase (GST) activity assay

Activity of glutathione-S-transferase (GST) in soluble (cytosol) and microsomal fraction was determined according to Habig (Habig et al., 1974) using the standard model substrate 1-chloro-

2.4-dinitrobenzene (cDNB). The reaction is initiated by addition of the enzyme to the assay mixture. The principle is based on the enhanced conjugation of the algicide (CDNB) on the sulphurhydryl (-SH) group of the co-substrate glutathione (GSH) present in the reaction mixture. The change in absorbance resulting from the conjugates formed was measured at 340 nm wavelength (extinction coefficient ε = 14.400 l/mmol x cm) for CDNB respectively. The GST assay mixture consisted of the following:

Glutathione-S-transferases (GST) assay

Reagent	**Volume (µl)**
NA_3PO_4-buffer (0.1 M, pH 6.5)	1100
Glutathione (GSH) (58.6 mM)	40
CDNB (29.6 mM)	40
Sample (enzyme) / H_2O (blank ref.)	40

$$1-chloro-2{,}4-dinitobenzene\ (CDNB) + \mathrm{GSH} \xrightarrow{\mathrm{GST\ (pH\ 6.5)}} GS-DNB\ conjugate$$

$$(\downarrow Abs = 340\ nm)$$

2.2.5.2 Calculation of enzyme activity

To measure the enzyme activity there is a convenient method of communicating of enzyme activity present in each milligram of protein. The catalytic activity of an enzyme is determined by the rate (velocity) of the catalysed reaction under optimum reaction conditions (optimum pH, temperature and substrate concentration high enough that maximum rate is reached). The rate of the reaction is defined by the substrate turn over per time unit and expressed as micromoles per second (µmol/s). The catalytic activity (CA) in a specific volume, which is a specific factor, is thus calculated as follows:

$$CA = \frac{\Delta e \times V \times 1000}{\varepsilon \times \mathrm{d} \times \Delta t \times v}$$

Δe = extinction change per minute; V = final volume of enzyme assay (µl); ε = substrate molar extinction coefficient (l / mmol x cm); d = cuvette width (cm); Δt = measuring time interval; v = volume of enzyme extract in assay (µl).

For the purpose to compare enzyme activities among treatments, CA was expressed as a specific factor of total protein concentration in the enzyme extract, thereby yielding a specific activity (SA).

$$SA = \frac{CA}{[\,Protein\,]}$$

[Protein] = protein concentration of enzyme extract (µg/µl)

A conventional unit for expressing enzyme activity is called katal. One katal is the amount of enzyme that catalyses the conversion of one mole of substrate per second. Enzyme activity was thus uniformly expressed in nanokatals per milligram protein (nkat/mg protein).

The multi-enzyme extracts were constantly kept on ice during all assays and were stored at – 80°C after shock freezing them in liquid nitrogen.

2.2.6 Statistics

Datasets were checked for homogeneity (Levene's test) and normality (Sharpirow-Wilk´s test) (Zar, 1996). Analysis of variance (ANOVA) was then expressed as means ± standard deviation and performed to test for differences among treatments. One-way ANOVA was used throughout in dose-response experiments. When ANOVA indicated significant differences, planned post-hoc comparisons or contracts were also performed using either Tukey's honest significant Difference (HSD) or Newman - Keuls' test. In cases where unequal numbers were involved, Tukey's HSD for unequal "n" test was used. All comparisons were made at the 5 % probability level of significance. The statistical analysis was performed using Statistica software (STATISTICAL Statsoft, Inc. 2002) and Origin (OriginLAB, OriginPro8G).

Significances for the bioaccumulation experiment with two different concentrations of PhACs (n=5) were calculated by ANOVA followed by Duncan´s Test (* $p<0.05$), and differences between the two treatments were tested by T-test (STATISTICAL Statsoft, Inc. 2002).

For the determination of the effect of PhACs on the biochemical parameters of *C. demersum,* significant differences between treatments (PhACs concentration) were assessed by one-way ANOVA followed by the Tukey post-hoc test (STATISTICAL Statsoft, Inc. 2002). All comparisons were also made at the 5 % probability level of significance.

3. Results

3.1 Uptake and bioaccumulation of PhACs in the non-target organism *C. demersum*

Compounds, even non-polar ones, are assimilated by intact plants. Uptake and bioaccumulation depended on the plant species and on the physico-chemical properties of the chemicals (Harms, 1992).

3.1.1 Carbamazepine

The starting concentration of the 0.9 x 10^{-4} mol/l CBZ was measured at 21.36 ± 3.23 mg/l CBZ in the medium. During static exposure, concentration was detected at 22.33 ± 1.31 mg/l at the first sampling point (1 d) and 19.83 ± 2.13 mg/l on the second (4 d). The concentration was added up to 8.56 ± 4.87 mg/l of CBZ on the last day of exposure (7 d). In the control samples was no CBZ detected.

Fig. 3.1 shows the applied concentrations of CBZ of both concentrations in the *C. demersum* tissue. The measurement of 0.9 x 10^{-4} mol/l CBZ concentration in the tissue was measured after 24 h at 65.57 ± 2.14 mg/l. On day seven the concentration had decreased to 19.43 ± 2.09 mg/l (Fig. 3.1).

The uptake and retention of PhACs from the exposure medium occurs via the whole macrophyte surface. The calculated BCF in *C. demersum* was 3.1-fold (1 d), 2.0-fold (4 d) and reached 1.0-fold (7 d), in the 0.9 x 10^{-4} mol/l CBZ concentration. A rapid bioaccumulation was observed shortly after the start of the exposure in *C. demersum* (1 d). Calculated BCF was then decreased for both CBZ concentrations.

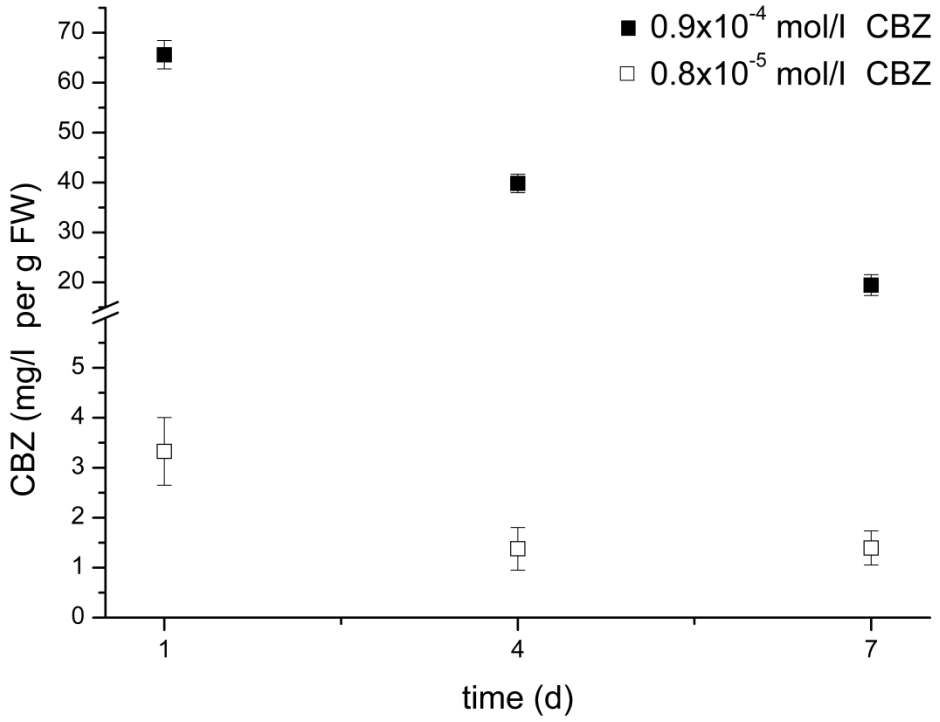


Fig. 3.1 Concentrations of Carbamazepine (CBZ) were measured in *C. demersum* via LC-MS/MS (mg/l per g FW) over the exposure time of 1, 4, and 7 days. Concentrations of 0.9 x 10^{-4} mol/l CBZ and 0.8 x 10^{-5} mol/l CBZ were used for the exposures.

The second tested concentration (0.8 x 10^{-5} mol/l CBZ) in the medium started with a value of 2.03 ± 0.11 mg/l CBZ, and decreased over the exposure time to 2.0 ± 0.11 mg/l (1 d), 1.24 ± 0.01 mg/l (4 d) and 1.1 ± 0.2 mg/l (7 d). Moreover, the maximal uptake in the FW of *C. demersum* reached up after 24 h (3.32 ± 0.67 mg/l CBZ) (Fig. 3.1). The end of the exposure, the concentration in the tissue reached up to 1.39 ± 0.34 mg/l CBZ. For the concentration of 0.8 x 10^{-5} mol/l CBZ, BCF was calculated at 1.6-fold at day one (1 d) and there was only a small difference between the 4 d and the 7 d of exposure (0.67 and 0.68 fold).

3.1.2 Ibuprofen

The exposure started with the applied concentration in the medium of 2.5 x 10^{-3} mol/l IBU which was 672.00 ± 387.50 mg/l of IBU. The concentrations were 623 ± 49.49 mg/l and 586 ± 55.24 mg/l of IBU on day 1 and day 4, respectively. Measured concentration in the medium had decreased to 526.66 ± 73.81 mg/l of IBU on 7 d. In none of the control samples could IBU be detected.

Compared to the other PhACs, the value of the anti-inflammatory drug IBU in the macrophytes *C. demersum* was lower during the first 24 h in the 2.5 x 10^{-3} mol/l IBU concentration Fig. 3.2. An increase of IBU was noticed on day 4 (283.83 ± 73.01 mg/l IBU). On day 7, the content was measured to be 159.50 ± 20.61 mg/l IBU. The uptake of IBU in *C. demersum* exposed to 2.5 x 10^{-3} mol/l IBU was measured on the 1d to be 0.11-fold. Interestingly on the fourth sampling day the value was 0.42 fold and decreased to 0.24-fold after one week (7 d) of exposure.

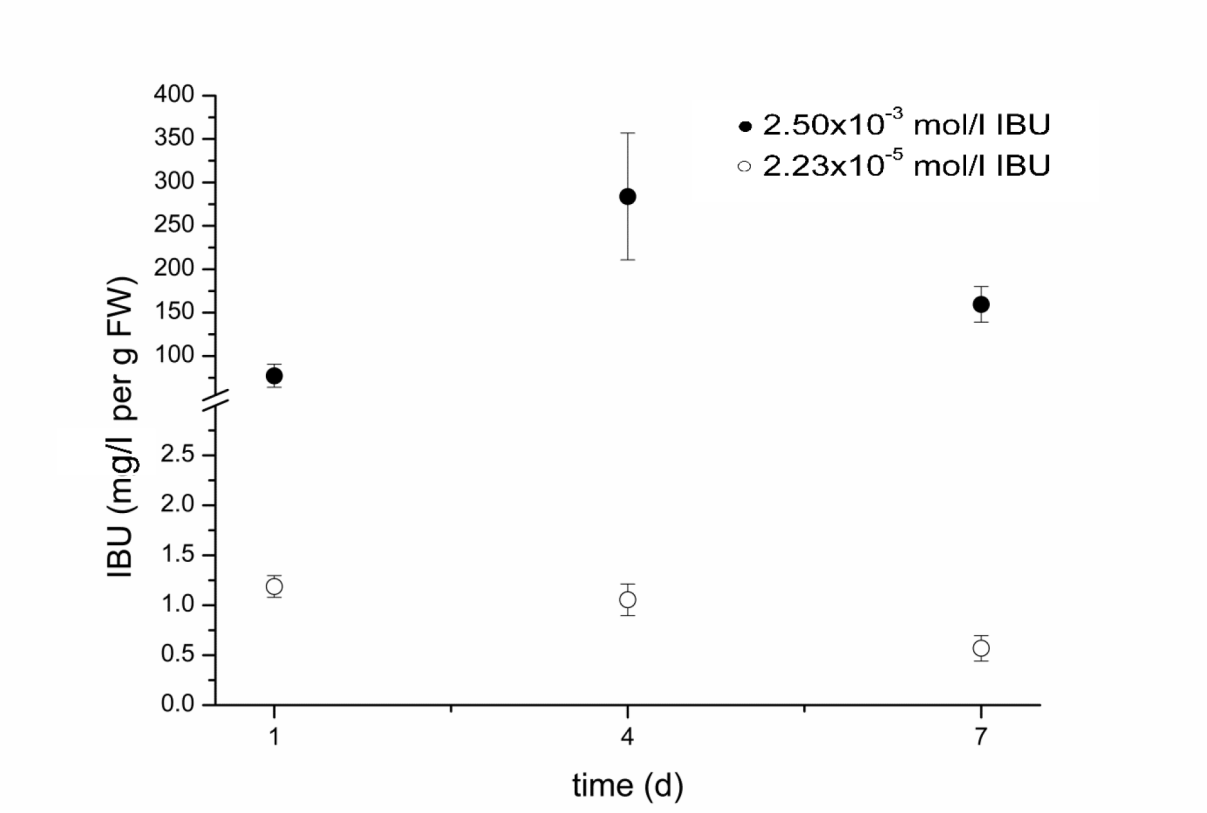


Fig. 3.2 Concentrations of Ibuprofen (IBU) were measured in the cells of the macrophyte *C. demersum* via LC-MS/MS (mg/l per g FW). The exposures were set at the concentration of 2.50 x 10^{-3} mol/l IBU and 2.23 x 10^{-5} mol/l IBU for 1, 4, and7 days.

During the one week of exposure, treatment concentrations of 2.23 x 10^{-5} mol/l IBU in the medium were measured at 2.33 ± 0.49 mg/l (1 d), 2.08 ± 0.42 mg/l (4 d) and 1.60 ± 0.22 mg/l (7 d) of IBU. The concentration of IBU in the macrophyte tissues showed a maximum of 1.18 ± 0.10 mg/l IBU (1 d) on the first sampling day and decrease to 0.58 ± 0.12 mg/l IBU (7 d) in *C. demersum* (Fig. 3.2). In contrast, the treatment with the 2.23 x 10^{-5} mol/l IBU concentration was bioaccumulated up to 0.57-fold in the macrophyte within the first day (1 d), then decreased to 0.42-fold (4 d), and even further to 0.22-fold (7 d).

Also during the exposure of the 2.23 x 10^{-5} mol/l IBU concentration, the OH-IBU content in the FW tissue of *C. demersum* was measured. On the first day we detected 0.004 ± 0.002 mg/l OH-IBU (1 d) per g FW content, and an increase was observed over the time of exposure (Fig. 3.2).

3.1.3 Levonorgestrel

Compared to the other PhACs (CBZ, IBU, and MET), concentrations of the medium of 33.10 ± 3.06 µg/l LNG (1.06 x 10^{-7} mol/l LNG) and 3.03 ± 0.15 µg/l LNG (0.97 x 10^{-8} mol/l) matched more closely the concentrations found in the environment. The 1.06 x 10^{-7} mol/l LNG concentration in the medium decreased from a value of 33.10 ± 3.06 µg/l to 0.31 ± 0.12 µg/l LNG over seven days of exposure. Similarly the 0.97 x 10^{-8} mol/l concentration of LNG decreased from 3.03 ± 0.15 µg/l to 0.0015 ± 0.0001 µg/l LNG (Fig. 3.3) over the duration of the exposure. It was no LNG concentration measurable in the control samples.

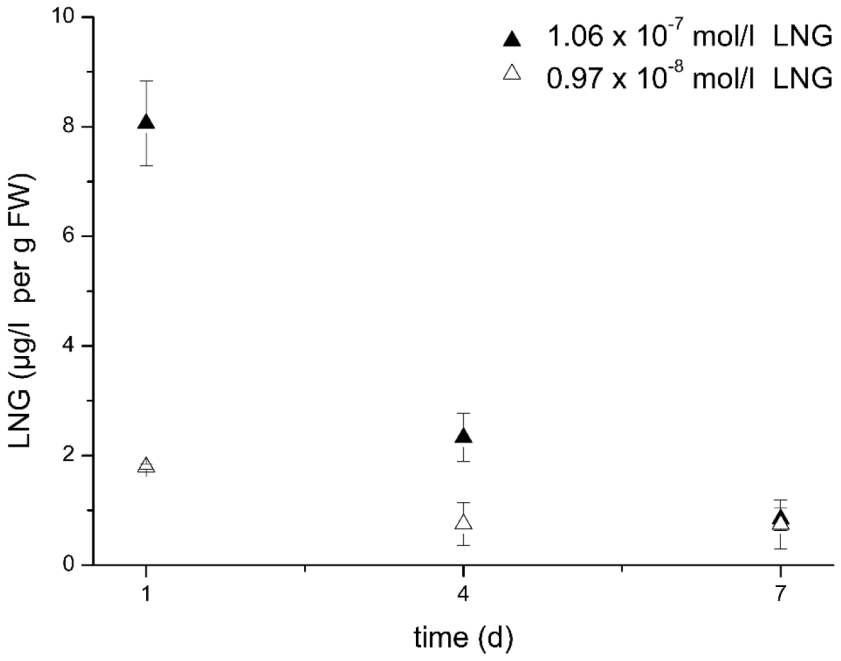


Fig. 3.3 Uptake concentrations of Levonorgestrel (LNG) measured in the cells of *C. demersum* via LC-MS/MS (µg/l per g FW). The exposure was performed over a period of 1, 4, and 7 days, using a concentration of 1.06 x 10^{-7} mol/l LNG and 0.97 x 10^{-8} mol/l LNG.

The maximum uptake of LNG in FW of *C. demersum* was reached after 24 h (Fig. 3.3). The concentration of 1.06 x 10^{-7} mol/l LNG in the macrophyte revealed on the first day 8.06 ± 0.78 µg/l LNG. Content decreased until the end of the exposure, to reach a value of 0.84 ± 0.21 µg/l LNG. Moreover, the maximum of the 0.97 x 10^{-8} mol/l uptake in FW of *C. demersum* was reached after 24 h. The exposure decreased to 0.75 ± 0.14 µg/l of LNG.

In the lower LNG concentration (0.97 x 10^{-8} mol/l) we detected a 0.649-fold uptake (1.79 ± 0.04 µg/l LNG) after 24 h, which was 2.45 times more compared to the higher concentration of (1.06 x 10^{-7} mol/l LNG; 0.265-fold). On the fourth day (4 d) the reduction was measured to be near to 50 % (0.330-fold). On the day 7 (d) the uptake content of both tested concentrations was nearly the same 0.84-fold (1.06 x 10^{-7} mol/l LNG) and 0.75-fold (0.97 x 10^{-8} mol/l LNG).

3.1.4 Metoprolol

At the start of the exposure, the applied concentration in the medium of 4.1 x 10^{-3} mol/l MET was 1091.33 ± 327.60 mg/l of MET. A rapid decrease in the medium was measured shortly after the start of the exposure (1 d) (1091.33 ± 327.60 mg/l MET) and kept on declining to reach a value of 359.80 ± 99.52 mg/l MET. During the time of exposure MET was not detected in any of the control samples.

Fig. 3.4 shows the results of MET – exposure in *C. demersum.* After 24 h of incubation with MET, macrophyte cells contained 239.97 ± 15.84 mg/l MET per g FW. The maximum content was 358.62 ± 56.23 mg/l MET on the fourth day (4 d). The data shows that MET is the second most bioaccumulated PhACs in the macrophyte *C. demersum.* Over the time of exposure, the calculated BCF decreased from 43.47-fold (1 d) to a 33.05-fold (4 d), and down to 17.89-fold (7 d).

The 1.8 x 10^{-5} mol/l MET concentration started in the medium with 4.81 ± 0.340 mg/l MET and decreased to 0.83 ± 0.14 mg/l MET within the first 24 h and further to 0.13 ± 0.051 mg/l MET. At this concentration, there was no measurable increase in the uptake of MET (7.91 ± 1.1 mg/l MET (1 d); 7.78 ± 1.8 mg/l MET (4 d); 7.95 ± 1.0 mg/l MET (7 d)) over time. However, BCF did reach a concentration of 1.8 x 10^{-5} mol/l MET (16.21-, 15.87- to 16.21-fold).

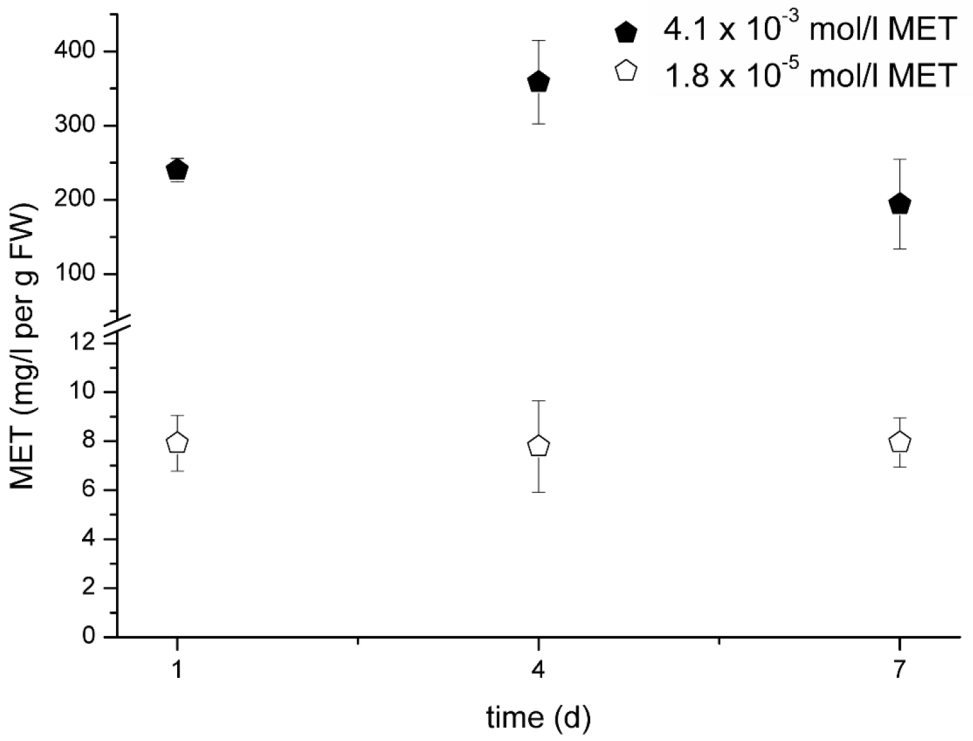


Fig. 3.4 Concentrations of Metoprolol (MET) in the macrophytes *C. demersum* were measured with LC-MS/MS (mg/l per g FW). Concentrations of 4.1 x 10^{-3} mol/l MET and 1.8 x 10^{-5} mol/l MET were used in the exposure and sampled on day 1, 4 and 7.

3.2 Metabolites of PhACs

Depending on the plant species, large amounts of chemicals and/or their metabolites, are frequently incorporated into non-extractable residues (Harms, 1992). Therefore, we couldn't detect metabolites of LNG and CBZ in the cells of *C. demersum*.

3.2.1 Metabolites of ibuprofen (hydroxyl-Ibuprofen)

Fig 3.5 illustrates the identified metabolite of IBU, OH-ibuprofen (OH–IBU). Initially OH–IBU was detected at the exposure concentration of 2.5 x 10^{-3} mol/l IBU at 0.001 ± 0.001 mg/l per g FW; and on day 4 (0.005 ± 0.002 mg/l per g FW). Over the duration of the experiment the content increased up to 0.008 ± 0.004 mg/l per g FW.

During the exposure of the 2.23 x 10^{-5} mol/l IBU concentration, a hydroxyl–ibuprofen (OH-IBU) content in the FW tissue of *C. demersum* was measured. On the first day, 0.004 **±** 0.002 mg/l OH-IBU (1 d) per g FW was measured and a subsequent increase over the

time of the exposure was then observed (Fig. 3.5). The metabolite OH-IBU could not be detected in the medium over the duration of the exposure.

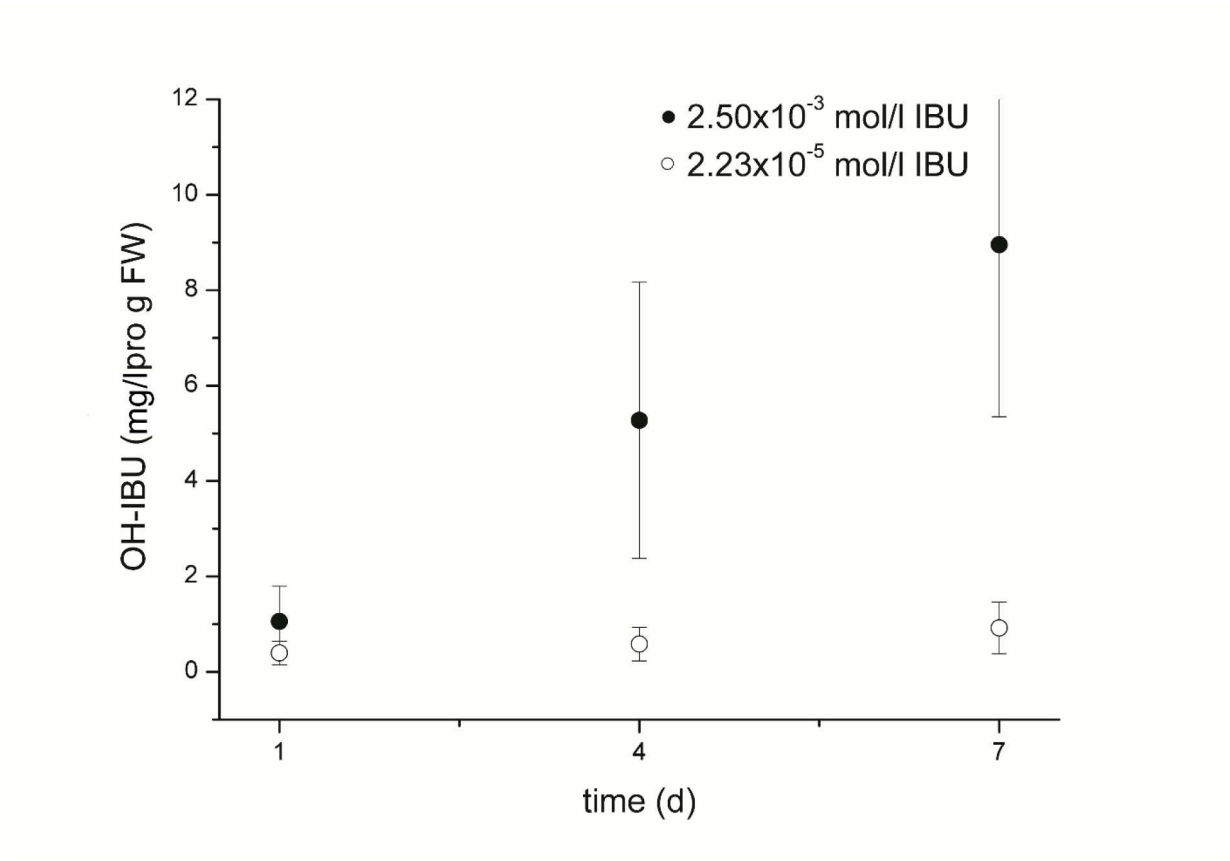


Fig. 3.5 The metabolites of ibuprofen, OH-ibuprofen (OH-IBU, Fig. 3.2) was measured in the cells of the submerged macrophytes *C. demersum* by LC-MS/MS (mg/l per g FW). The exposures were set at the concentrations of 2.50 x 10^{-3} mol/l IBU and 2.23 x 10^{-5} mol/l IBU over 1, 4, and 7 days.

3.2.2 Metabolites of metoprolol (O-desmethyl-metoprolol)

After 24 h with the 4.1 x 10^{-3} mol/l MET concentration, it was not possible to measure metabolites. On the second sampling day (4 d), it was possible to detect one metabolite of MET, namely *O*-desmethyl-metoprolol (*O*–desmethyl-MET) (130.66 ± 16.46 mg/l MET) (Liang et al., 2010). Moreover, over the course of the experiment the content of this metabolite increased (Fig. 3.6). Also as in the case of OH–IBU, we did not find this metabolite in the treated medium.

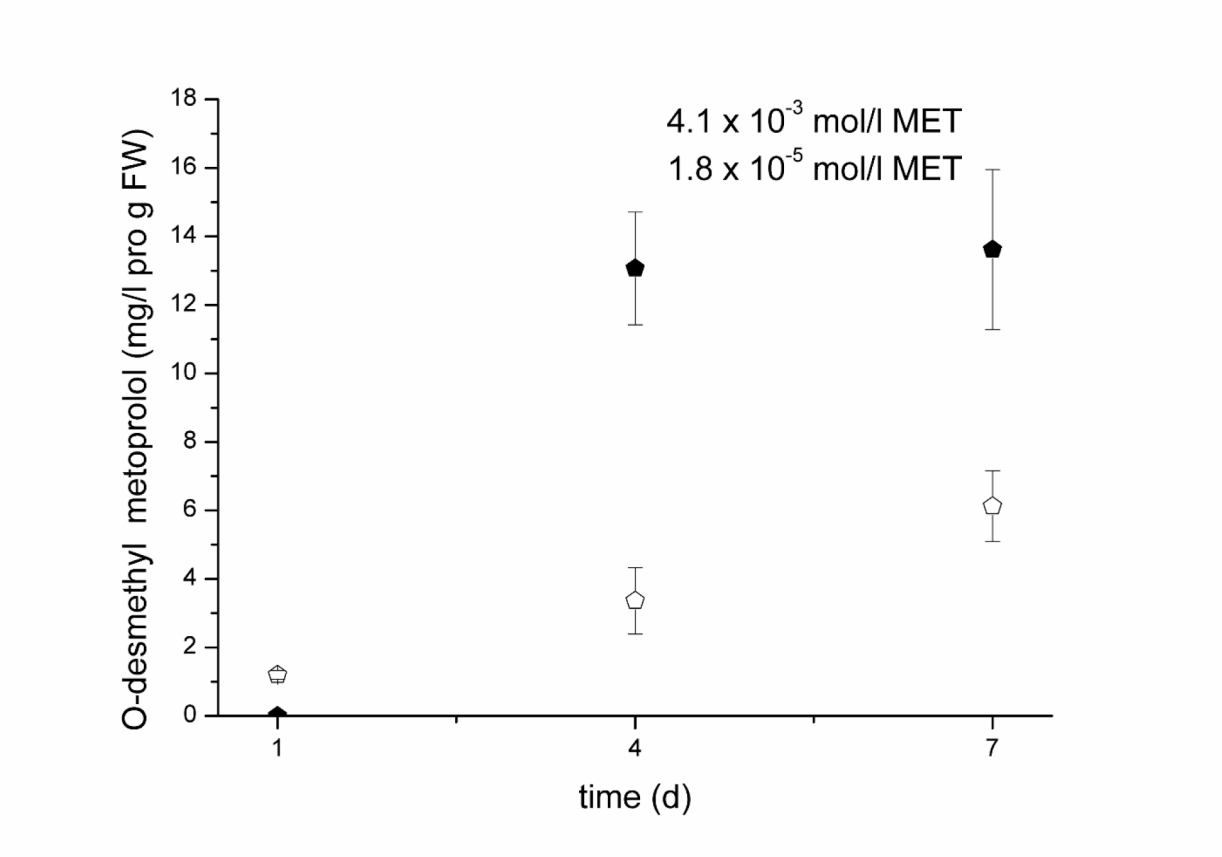


Fig. 3.6 Concentrations of the metabolite of metoprolol: *O*–desmethyl-metoprolol (*O*- desmethyl- MET) were measured by LC-MS/MS (mg/l per g FW). The concentrations of 4.1 x 10^{-3} mol/l MET and 1.8 x 10^{-5} mol/l MET were used for exposure; and sampled on days 1, 4 and 7

3.3 Time - response effect experiment of PhACs on *C. demersum*

ROS production and subsequent damage are an important mechanism of toxicity in organisms exposed to xenobiotics. The macrophyte species *C. demersum* appeared to be healthy from the outside with no signs of necrosis, chlorosis or wilting observed after 1 d and 7 d exposure to the different PhACs concentrations. The concentrations used in this experiment differ from the bioaccumulations experiments.

3.3.1 Cabamazepine

The exposure concentrations of CBZ were of 23.62 µg/l (1 x 10^{-7} mol CBZ) and 2362.70 µg/l (1 x 10^{-5} mol CBZ) in the medium for 7 days. Evidence of an inhibitory effect was not found on the activity of catalase (CAT) of CBZ (Fig. 3.7A). An increased activity of CAT was detected at the concentration of 10^{-5} mol CBZ (159.79 ± 7.61 nkat/mg protein) in *C. demersum* after 7d exposure. The differences were not significant due to the standard deviation of the control (115.07 ± 47.78 nkat/mg protein).

The activity of guaiacolperoxidase (POD) showed an increase in both tested concentrations at 24 h. In the 10^{-5} mol CBZ concentration the increase was up to 1.7 fold higher (64.19 ± 9.73 nkat/mg protein) compared to the control (36.76 ± 11.99 nkat/mg protein). Furthermore, for the concentration of 10^{-7} mol CBZ, also an activity increase with a high standard deviation (53.86 ± 35.61 nkat/mg protein) was measured. In the solvent control solutions, no significant changes were observed at any time point. A dose-dependence in POD activity was not measured for both concentration of CBZ after 7 d exposure (Fig. 3.7B).

The membrane bound GST activities (mGST) were decreased in contrast to the soluble GST (sGST) (10^{-5} mol CBZ 0.21 ± 0.04 nkat/mg protein; control 0.37 ± 0.18 nkat/mg protein); no significances were found (Fig. 3.7C). Further concentration dependent decreases of mGST activity were observed, but a high standard deviation of the control sample did not allow significance (0.45 ± 0.28 nkat/mg protein).

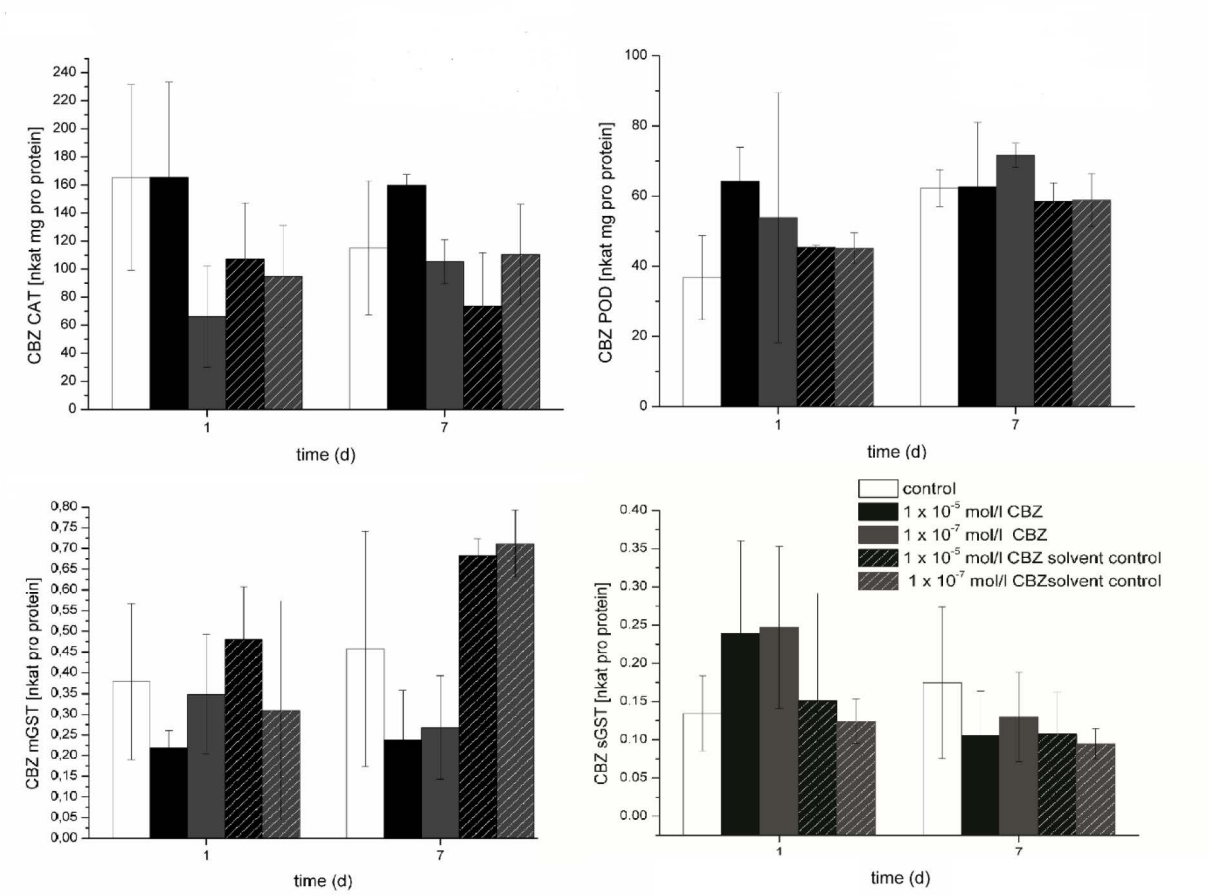


Fig. 3.7 Enzymatic response of catalase (A), peroxidase (B), glutathione-S-transferase (mGST; C), and (sGST;D) of *C. demersum* after exposure with 10^{-5} and 10^{-7} mol/l of Carbamazepine (1d and 7d). Significant differences to controls can be noted for every treatment ($P < 0.05$).

The measurements in the biotransformation activity with the two concentrations of CBZ were shown not to be significantly different for *C. demersum*. Increases with high standard deviation were noticed for sGST in both concentrations of CBZ (0.23 ± 0.12 and 0.24 ± 0.11 nkat/mg protein) at day 1 (Fig. 3.7D).

3.3.2 Ibuprofen

During the exposure of the macrophyte with IBU (1 x 10^{-7} mol/l IBU and 1 x 10^{-5} mol/l IBU) a tendency towards enzyme activity decrease was observed. For both concentrations, the enzyme activity of CAT was measured at 33.64 ± 24.11 and 33.53 ± 19.55 nkat/mg protein (control 100.11 ± 49.90 nkat/mg protein) in the first 24 h. Therefore the enzyme activity of 10^{-5} mo/l IBU decreased to 2.06-fold (41.16 ± 17.85 nkat/mg protein; control 84.90± 17.27 nkat/mg protein) (Fig. 3.8A).

The POD activity decreased significantly in *C. demersum* (65.78 ± 31.63 nkat/mg protein; 10^{-7} mol/l IBU) after 1 d of exposure. The decrease was not significant for the concentration of 10^{-5} mol/l IBU (98.65 ± 23.77 nkat/mg protein; control 137.18 ± 13.09 nkat/mg protein). Compared to the control, 10^{-5} mol/l IBU was shown an inhabitation at the end of the exposure (7d) (100.84 ± 30.29 nkat/mg protein) (Fig. 3.8B).

The mGST content showed a significant decrease in enzyme activity in *C. demersum* at 10^{-5} mol/l IBU (0.08 ± 0.02 nkat/mg protein, control 0.45 ± 0.06 nkat/mg protein, Fig. 3.8C). The decreased enzyme activity was still present at day 7 (0.14 ± 0.05 mol/l of IBU).

The sGST showed no significant changes either after 1 d or at the end of the exposure (Fig. 3.8D).

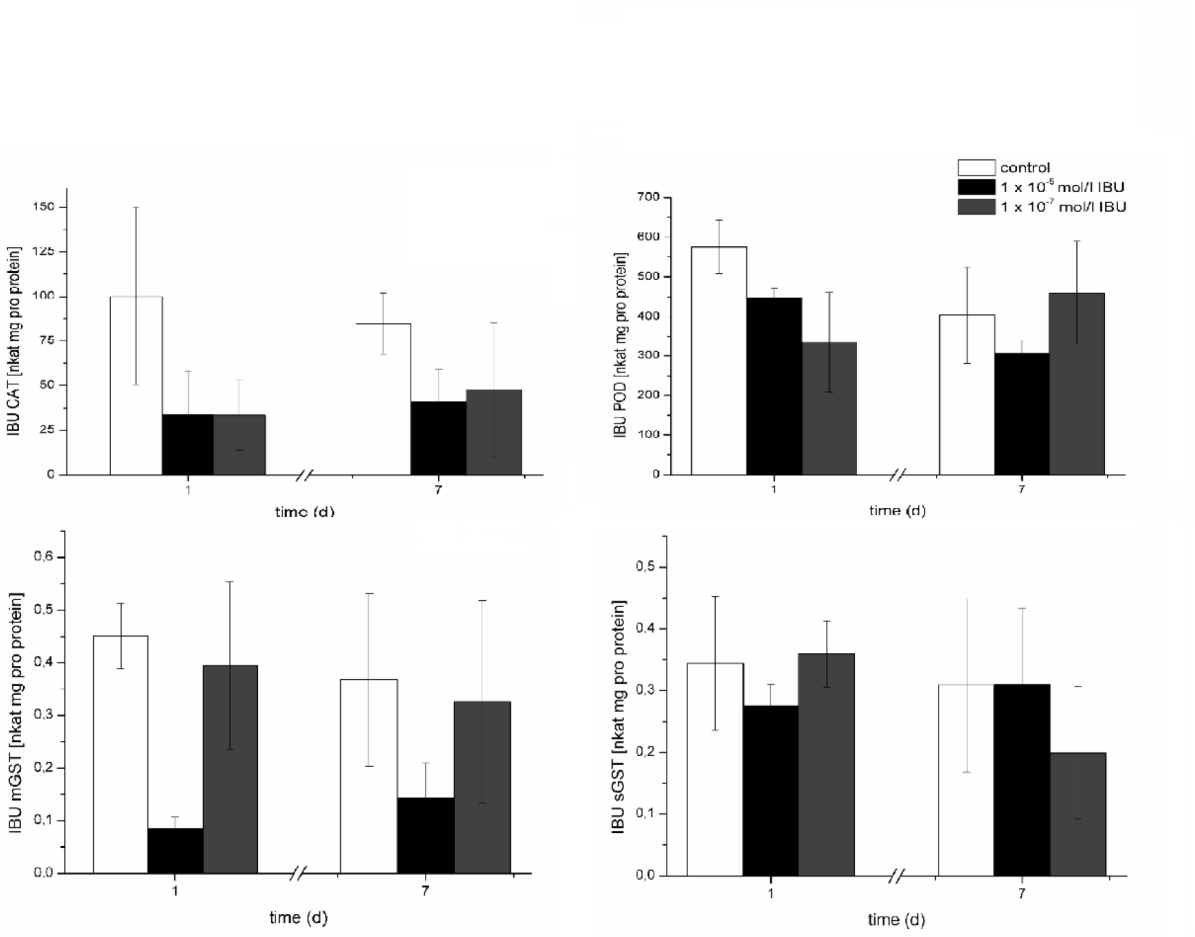


Fig. 3.8 Enzymatic response of catalase (A), peroxidase (B), glutathione-S-transferase (mGST; C), and (sGST;D) of *the macrophyte species C. demersum* after exposure to 10^{-5} and 10^{-7} mol/l of ibuprofen (1 d and 7 d). Significant differences to controls can be noted for every treatment ($P < 0.05$).

3.3.3 Levonorgestrel

The CAT activity decreased significantly in both tested concentrations 0.97 x 10^{-8} mol and 1.06 x 10^{-7} mol/l LNG (35.95 ± 15.34 nkat/mg protein, 10^{-8} mol/l LNG; 24.25 ± 12.72 nkat/mg protein, 10^{-7} mol/l LNG) after 24 h exposure. The exposure with 10^{-7} mol/l LNG was significantly reduced CAT enzyme activity after 7 d exposure (105.48 ± 43.43 nkat/mg protein; control 232.42 ± 41.79 nkat/mg protein) (Fig. 3.9A).

POD activity for 10^{-7} mol/l LNG concentration (Fig. 3.9B) was lower compared to the control samples (10^{-7} mol/l LNG 10.87 ± 3.41 nkat/mg protein; control 16.43 ± 4.71 nkat/mgprotein). The exposure concentrations of 10^{-8} mol/l LNG were measured as 8.30 ± 2.73 nkat/mg protein at 24 h. POD decreased significantly for the 10^{-7} mol/l LNG concentration (9.17 ± 3.31 nkat/mg protein; control 22.60 ± 2.74 nkat/mg protein) at day 7. The control containing also ethanol, which is acting as a baseline toxin, had the same result than the control samples.

In addition, the enzyme activity of sGST was not inhibited after 24 h. (Fig. 3.9C). The exposure of LNG over 7 d showed a concentration dependent response of the sGST activities. This included a strong decrease for the lower LNG concentration (0.04 ± 0.001 nkat/mg protein) and a tendency to decrease for 10^{-8} mol/l LNG (0.16 ± 0.03 nkat/mg protein).

The mGST response in *C. demersum* showed no significant changes after day 1; although a slight decrease for the 10^{-7} mol/l LNG concentration 0.11± 0.03nkat/mg protein, control 0.28 ± 0.17 nkat/mg protein) was noticed (Fig. 3.9D).

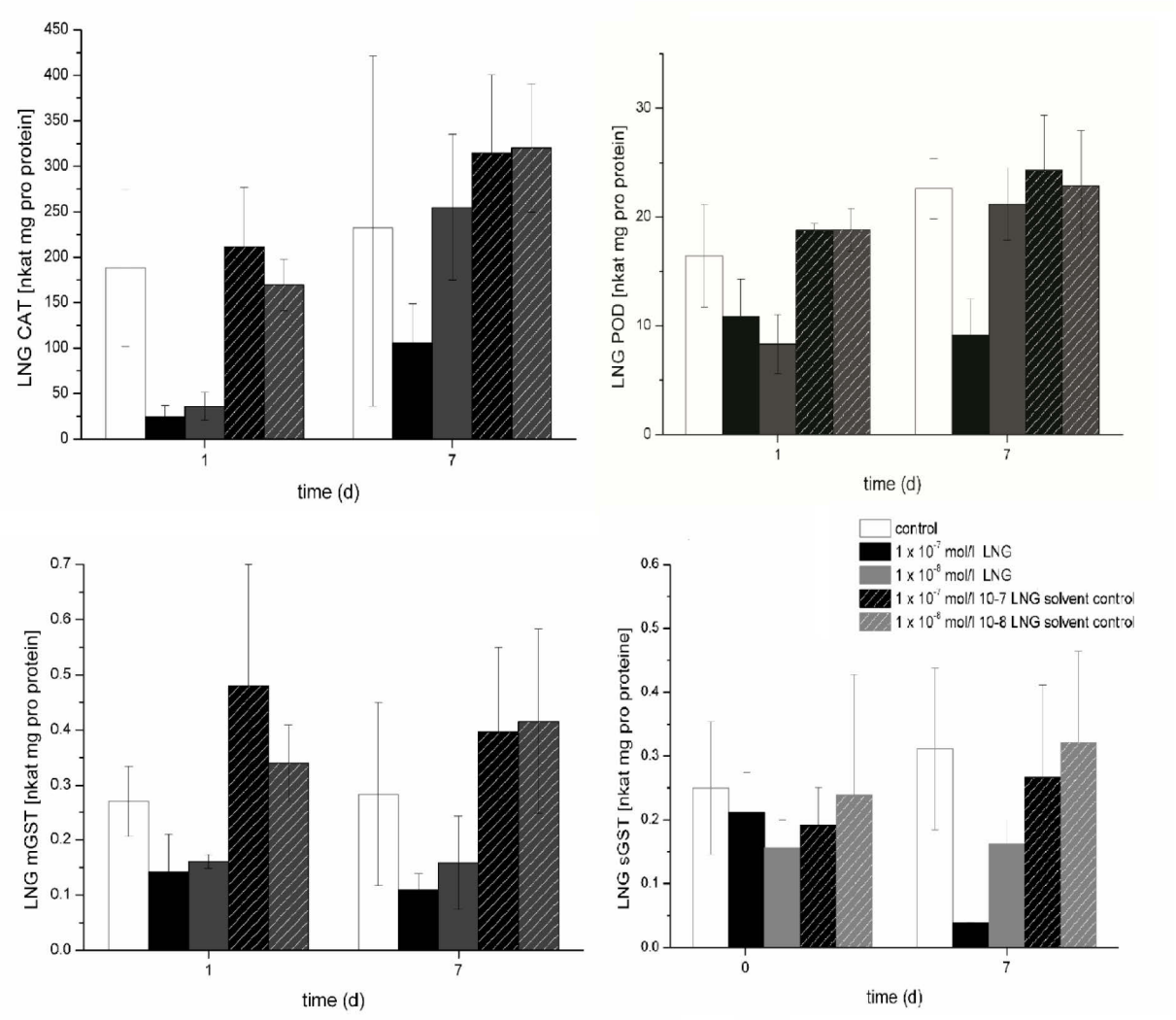


Fig. 3.9 Enzymatic response of catalase (A), peroxidase (B), glutathione-S-transferase (mGST; C), and (sGST;D) of *C. demersum* after exposure for 10^{-5} and 10^{-7} mol/l of Levonorgestrel (1 d and 7 d). Significant differences to controls can be noted for every treatment ($P < 0.05$).

3.3.4 Metoprolol

On both days of sampling there were no changes observed in the macrophyte tissues for CAT activity in the tissue, in both tested concentrations (10^{-5} mol MET and 10^{-7} mol MET) (Fig. 3.10A).

Within 24 h after exposure, the specific activity of POD decreased more than 56-fold and 50-fold, respectively, (10^{-5} mol/l MET 84.82 ± 11.38 nkat/mg protein; 10^{-5} mol/l MET 89.81 ± 36.58 nkat/mg protein) compared to the control concentration (184.52 ± 51.35 nkat/mg protein) (Fig. 3.10B). The further exposure showed no significance and no concentration dependent response after 7 d.

The sGST showed no significant activity after 24 h but with a decreased activity (0.39± 0.22 nkat/mg protein and 0.26 ± 0.23 nkat/mg protein). Compared to the other tested PhACs there was still a difference in sGST activity on day 7. The concentration 10^{-5} mol/l MET was slightly decreased (0.24 ± 0.09 nkat/mg protein). An increasing tendency at the 10^{-7} mol/l MET concentrations was measured (0.55 ± 0.19 nkat/mg protein, control 0.39 ± 0.25 nkat/mg protein) (Fig. 3.10D) at the end of the exposure.

The response of mGST in *C. dermersum* was similar to the sGST in both tested concentrations (10^{-5} mol/l MET 0.20 ± 0.02 nkat/mg protein; 10^{-5} mol/l MET 0.24 ± 0.06 nkat/mg protein, control 0.37 ± 0.19 nkat/mg protein) (Fig. 3.10C). Day 7 of the exposure the enzyme activities of mGST slightly increased at both concentrations.

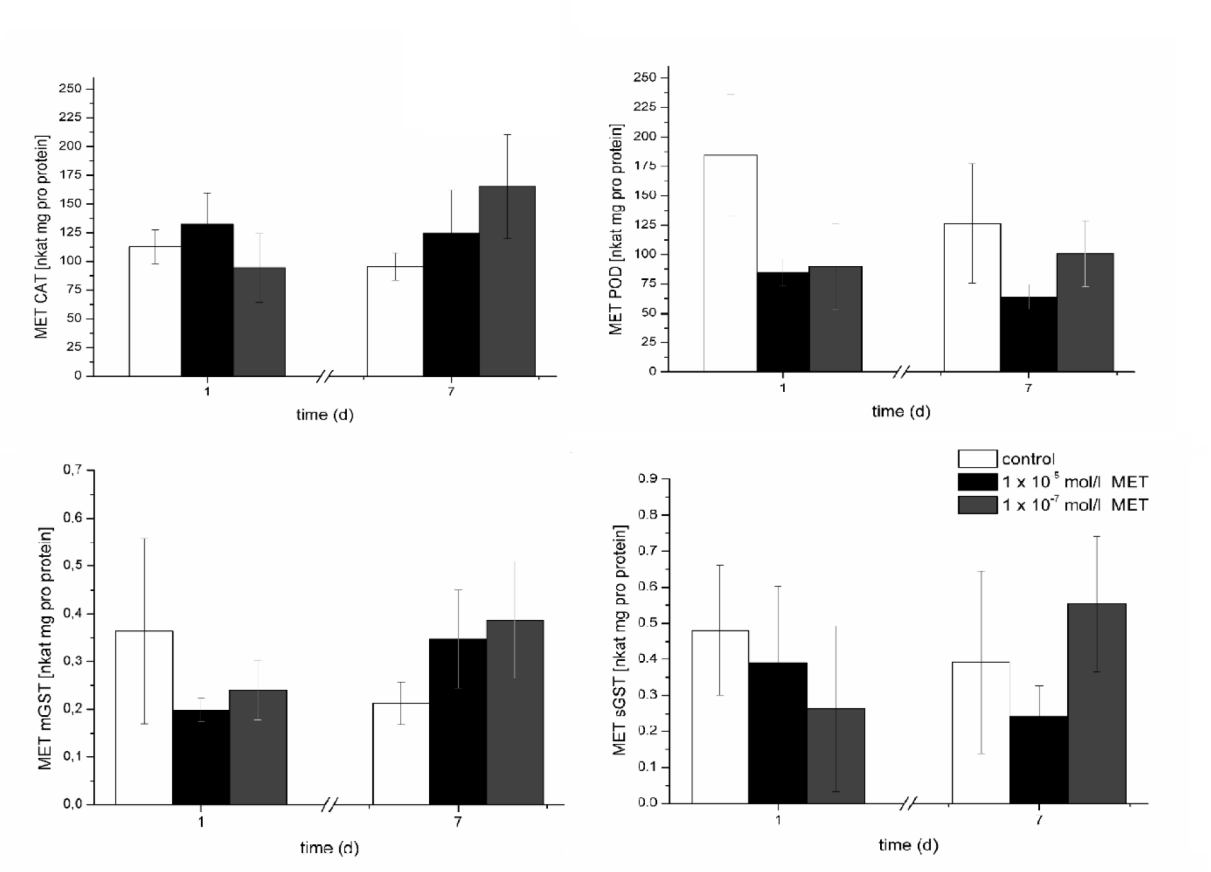


Fig. 3.10 Enzymatic response of catalase (A), peroxidase (B), glutathione-S-transferase (mGST; C), and (sGST;D) of *C. demersum* after exposure to 10^{-5} and 10^{-7} mol/l of Metoprolol for different durations (1 d and 7 d). Significant differences to controls can be noted for every treatment ($P < 0.05$).

3.4 Chlorophyll pigments of PhACs in *C. demersum*

Although the effect of the different factors are often indirect and not easily quantified or distinguished from one another; fluorescence measurements have successfully been used to monitor and characterize a wide variety of photosynthetic events.

3.4.1 Carbamazepine

During the one week exposure with the two different CBZ media (0.9 x 10^{-4} mol/l CBZ and 0.8 x 10^{-5} mol/l CBZ) *C. demersum* manifested no detectable effects on chlorophyll pigments (Fig. 3.11). All three chlorophyll contents tested (Chlorophyll a, Chlorophyll b and Chlorophyll total content) remained unchanged at all tested CBZ concentration, compared to control.

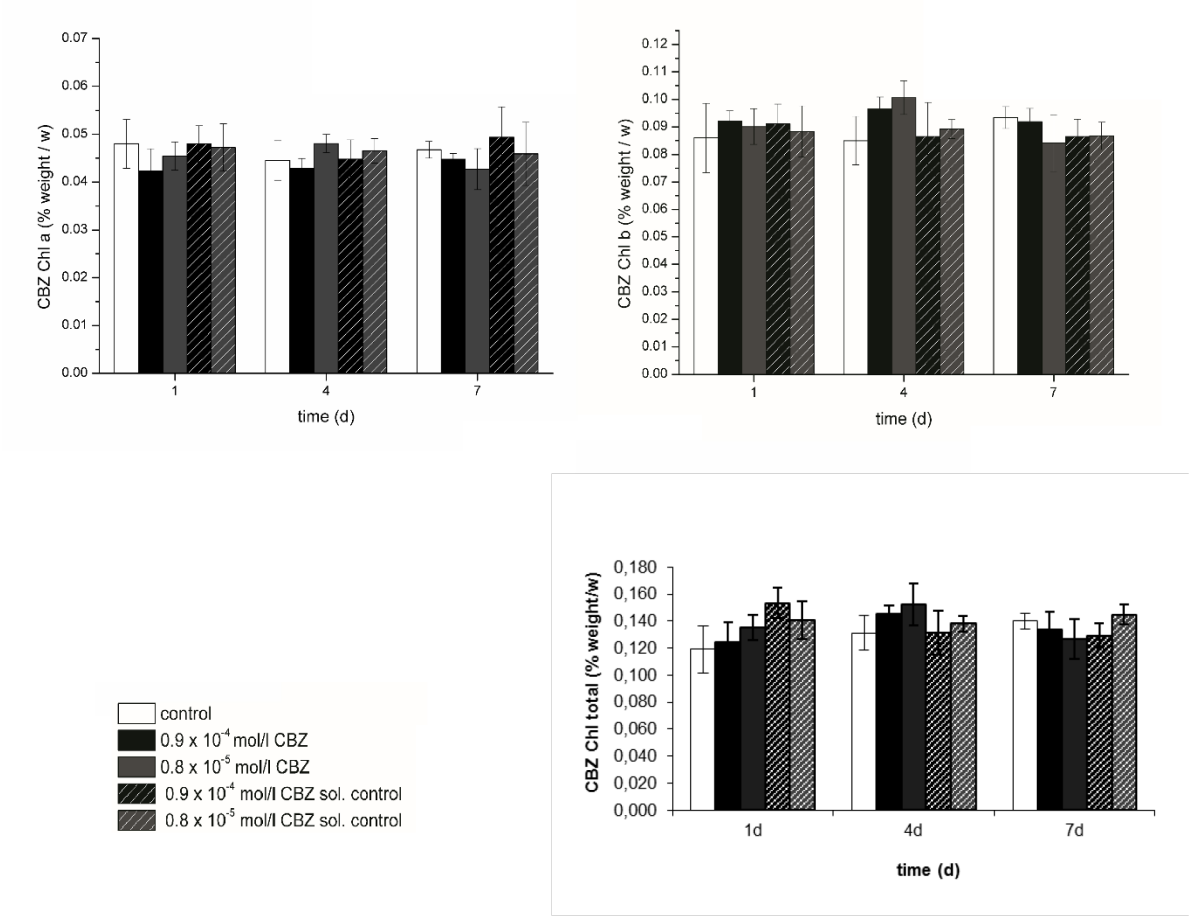


Fig. 3.11 Dose-response chlorophyll a, b and total (% weight /w) in *C. demersum* exposed to various CBZ concentration (mg/l). Bars represent mean (n = 5) ± standard deviation (SD), $p < 0.005$ (Newman-Keuls test)

3.4.2 Ibuprofen

Whilst monitoring the chlorophyll pattern of *C. demersum*, a switch in the ratio of both concentrations tested (2.5 x 10^{-3} mol/l IBU and 2.23 x 10^{-5} mol/l IBU) was observed. In comparison to the control, there was no change noticeable at 2.23 x 10^{-5} mol/l IBU. At 2.5 x 10^{-3} mol/l IBU there was a slight increase in all chlorophyll ratios measured in the first 24 h. In turn chlorophyll pigments of *C. demersum* showed a tendency towards inhibition for the higher concentration on the day 4 and 7 (Fig. 3.12).

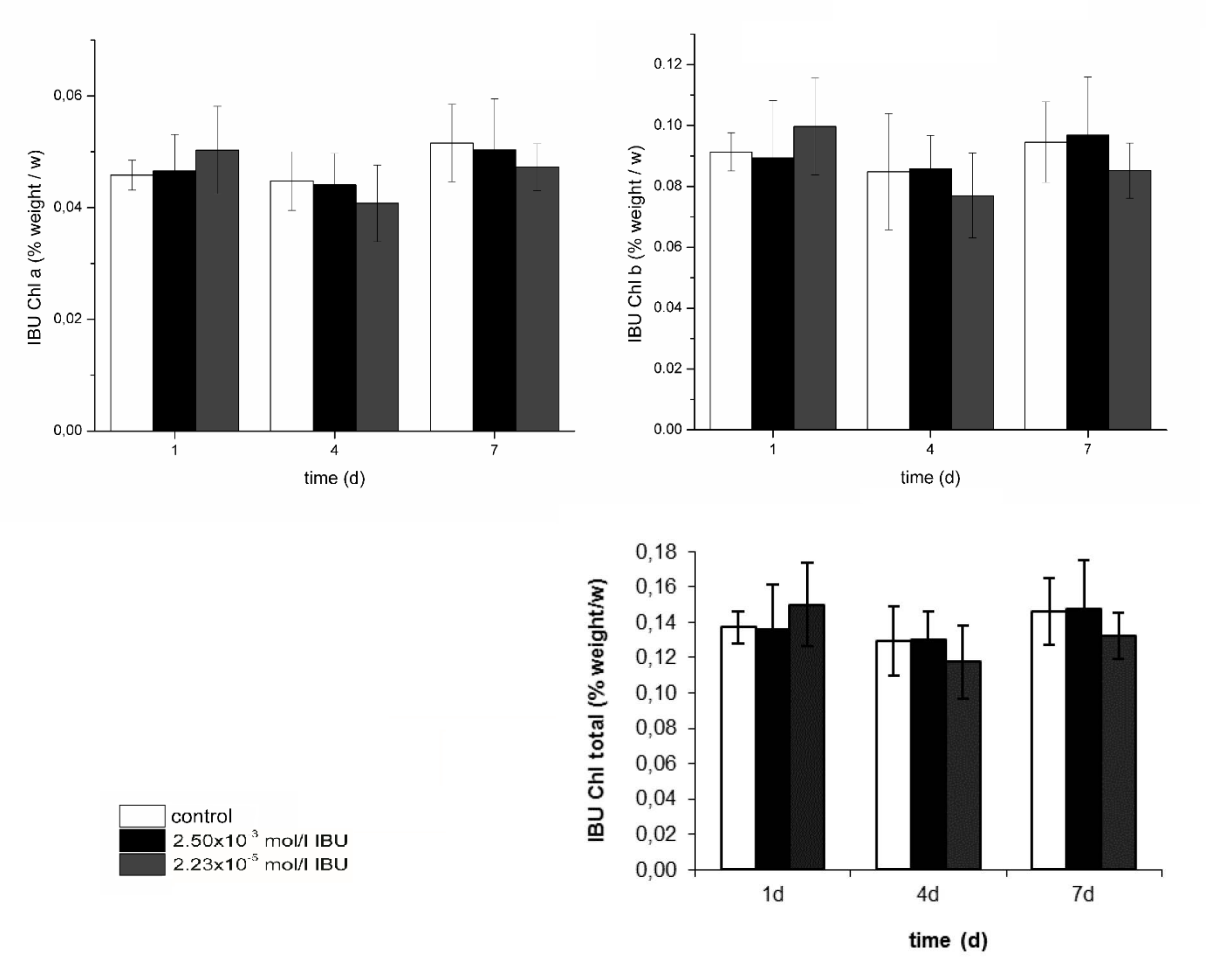


Fig. 3.12 Dose-response chlorophyll a, b and total (% weight /w) in *C. demersum* exposed to various IBU concentration (mg/l). Bars represent mean (n = 5) ± standard deviation (SD), p < 0.005 (Newman-Keuls test)

3.4.3 Levonorgestrel

During the exposure with two environmentally relevant LNG concentrations (1.06 x 10^{-7} mol/LNG and 0.97 x 10^{-8} mol/l LNG), *C. demersum* manifested no detectable effects on the chlorophyll a pigments (Fig. 3.13). The chlorophyll contents in Chl b changed at 1.06 x 10^{-7} mol/l LNG concentration at day 4 d, compared to control. The same switch of Chl total content could be found after exposure to LNG.

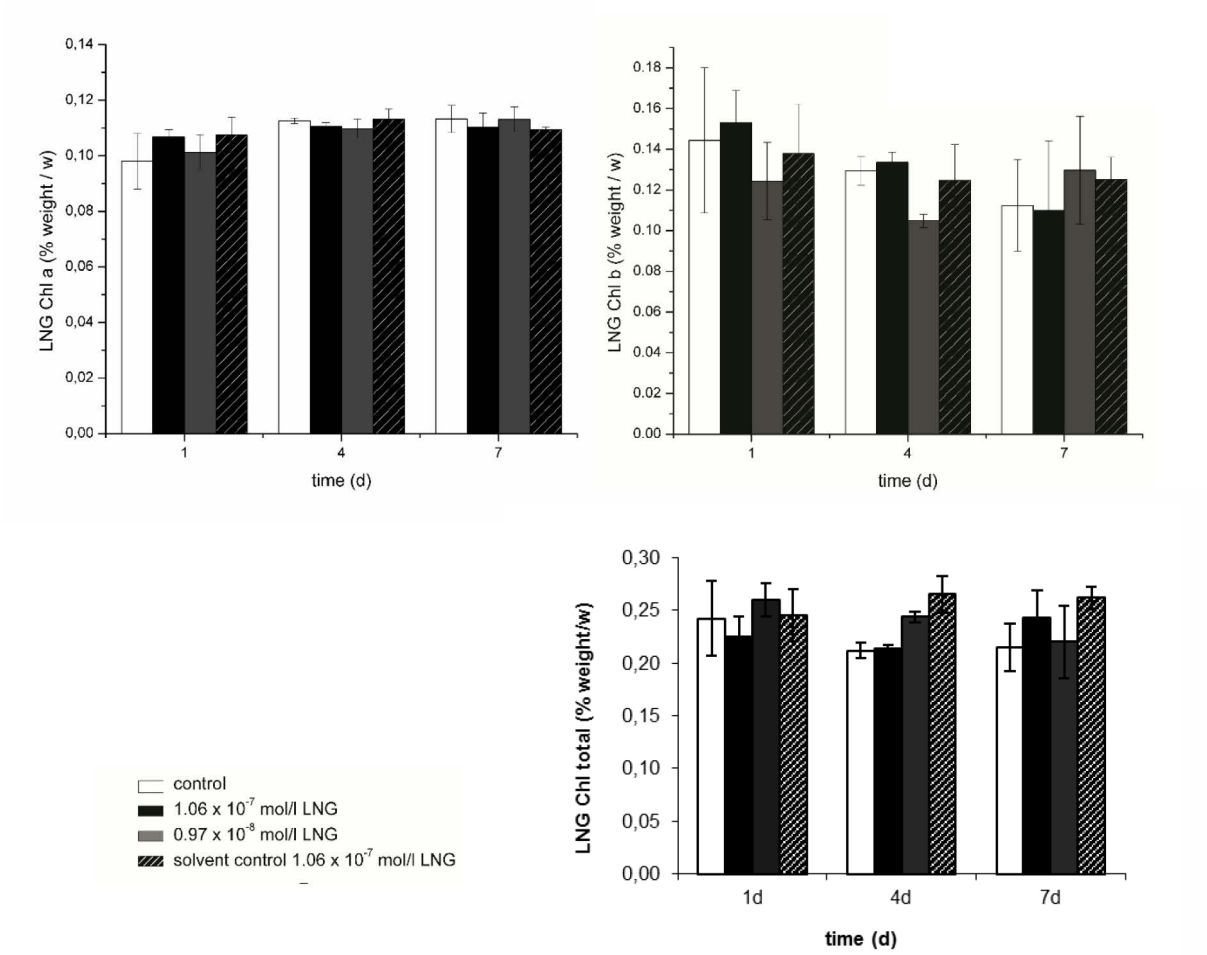
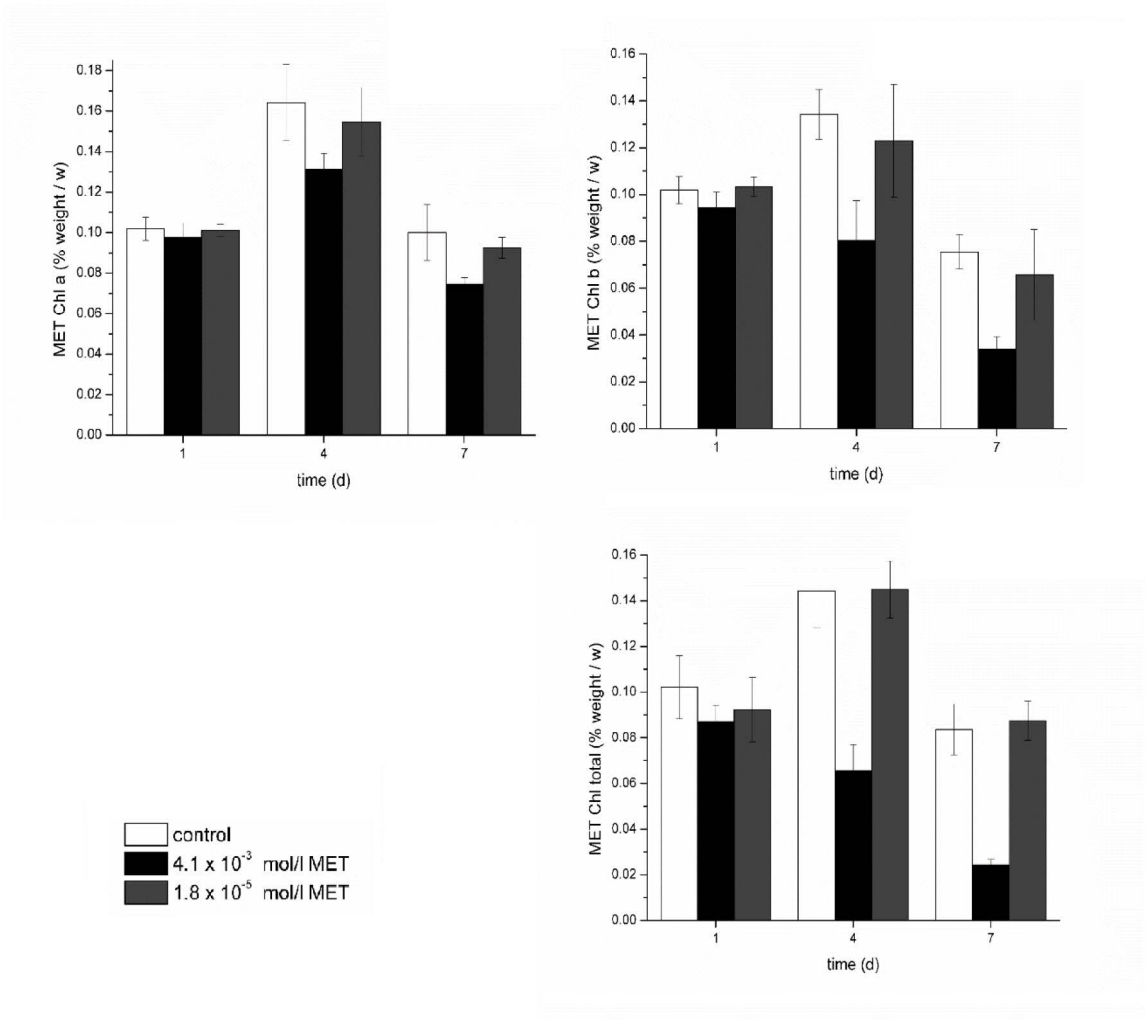


Fig. 3.13 Dose-response content of chlorophyll a, b and total (% weight /w) in *C. demersum* exposed to various LNG concentration (µg/l). Bars represent mean (n = 5) ± standard deviation (SD), $p < 0.005$ (Newman-Keuls test)

3.4.4 Metoprolol

Chlorophyll pigments of C. demersum were inhibited by MET at the higher concentration on day 4 and 7 (Fig.3.14). At 4.1 x 10^{-3} mol/l MET the pigments are significantly decreased on these two days by 45% and 29% ($p < 0.05$). After treatment with the non-environmentally relevant concentration of MET, it was noticeable that the tissue of C. demersum showed weak conditions. The pigment bleaching from dark green to pale yellow was observed, as well as the shedding of leaves.

Fig.



3.14 Dose-response chlorophyll content of Chlo a, b and total (% weight /w) in *C. demersum* exposed to various MET concentration (mg/l). Bars represent mean (n = 5) ± standard deviation (SD), $p < 0.005$ (Newman-Keuls test).

The detection of the chlorophyll pigments at the concentration of 1.8 x 10-5 mol/l MET showed a tendency towards inhibition also on day 4 and 7, in comparison to the control values and to the solvent control mediums values. Hence, the total chlorophyll content at this concentration remained unchanged for all tested days.

4. Discussion

4.1 Uptake and Bioaccumulation of PhACs in *C. demersum*

The reason why medical substances are relevant for the environment is that they are developed with the intention to cause biological effects. Therefore, it is interesting to evaluate what happens with the active substances. The results of the present study strongly suggest that PhACs bind to the cell membranes or are stored into the cells of the submerged macrophyte *C. demersum* and implies their uptake. Two of the tested substances (CBZ and MET) were bioaccumulated, and we were also able to find first step metabolites for two of the tested PhACs (OH-IBU and *O*-desmethyl-MET).

The concentrations of PhACs used for exposure were higher than previously described environmental concentrations. Only one of the tested compounds, LNG, was equal to the detection limits in the environment (de Alda et al., 2002; Vulliet et al., 2007).

The concentrations of PhACs in the medium decreased over time, which may have two possible explanations. The available active compound in the medium governed the amount of the chemical, which was taken up by the plants. The difference in the medium concentration of the control samples showed that the PhAC concentrations are still higher than the medium with the exposed macrophytes. This effect was also described by Nimptsch et al. (Nimptsch et al., 2008) for the cyanotoxin microcystin. Furthermore, an effect of photo-degradation of the medical substance could lead to decreased amounts of PhACs. Matamoros and colleagues (Matamoros et al., 2008) described this effect in a study.

Plants have nearly the same detoxification metabolism as animals, but they have no effective excretion pathway. Instead of possessing an excretion pathway they store the PhACs and xenobiotics in their vacuoles (soluble conjugates) and cell walls (most of the insoluble conjugates) (Sandermann, 1992). Furthermore, it is known that macrophytes reacted strongly to a single application of a highly effective biocide irgarol (Mohr et al., 2008) and also of natural toxin in *C. demersum* (Esterhuizen-Londt et al., 2011; Pflugmacher et al., 2001). Therefore, the effects of detoxification can explain the PhACs degradation in the plant.

PhACs, often have similar physico-chemical properties, e.g. they are lipophilic in order to be able to pass membranes; they may have a different ionization state to the chemical itself (Delépée et

al., 2004; Voutsas et al., 2002); or they are persistent in order to prevent inactivation before having a curing effect on other harmful xenobiotics. Thus the medical substances have many properties that can lead to bioaccumulation and thus provoking effects in aquatic ecosystems.

4.1.1 Carbamazepine

Two out of four tested PhACs (CBZ and MET, Fig. 3.1 and Fig. 3.4) were found to bioaccumulate in the macrophyte. The BCF of 0.9×10^{-4} mol/l CBZ calculated for the tissue were 26.1-fold, 20.4-fold, and 10-fold over time. Also, Vernouillet and colleagues (Vernouillet et al., 2010) showed in their study, that the green algae *Pseudokirchneriella subcapitata* bioaccumulated CBZ with a factor of 2.2 after 24 h, which is not as high as that on for *C. demersum*.

The bioaccumulation of CBZ in different parts of the cucumber where shown in a study (Shenker et al., 2011). The main concentration was measured in fruits, which could be an interesting point to take a closer look at fruit bodies of macrophytes. The process of CBZ toxicity highlighted the transfer between the trophic levels in aquatic organisms. It appears that most drugs are metabolized by cytochromes of the P450 3A family (Stresser et al., 2000). It would therefore be interesting to invest further work on the enzymatic effects of PhACs.

4.1.2 Ibuprofen

Compared to CBZ, the anti-inflammatory drug IBU did not reach its maximum of the concentration in *C. demersum* during the first 24 h. We were also not able to detect any bioaccumulation, therefore microbial removal and degradation could be the reason, which Langenhoff and colleagues showed in their study for IBU and diclofenac (Langenhoff et al., 2013).

In a previous study, Palmgrén and colleagues demonstrated a low adsorption of IBU and diclofenac to the surfaces (Palmgrén et al., 2006). At the concentration of 2.23×10^{-5} mol/l IBU the uptake values were found to be 0.6-fold, 0.48-fold and 0.22-fold. These values are in the same range with our results of the other tested PhACs.

Very low concentrations may result in effects in the field, or by effects in organisms at higher trophic levels, which Ericson and colleagues showed in their study of Baltic sea mussels (Ericson et al., 2010). In mammals, IBU and related NSAIDs are known to inhibit the cyclooxygenase (COX) pathway. NSAIDs mode of action in macrophytes could be similar to that of mammals or invertebrates. This is supported by evidence of inhibition of COX by NSAIDs in a wide range of

different species (e.g. Heckmann et al., 2007). Only at the highest concentration a decrease of the BCF within one week could be observed, which may hint at enhancing regulatory processes, e.g. metabolization.

4.1.3 Levonorgestrel

A number of studies are focused on the potential adverse environmental effects of steroids and other estrogens, since their interference with the endocrine responses of aquatic organisms was discovered (Routledge et al., 1998; Svensson, 2010). In the present study the concentration of 0.97×10^{-8} mol/l LNG was taken up 0.59-fold, whereas *C. demersum* exposed to the higher concentration (1.06×10^{-7} mol/l LNG) displayed a much lower BCF of 0.24-fold. This is in accordance with a previous study on *Dreisena polymorpha* by Contardo–Jara et al. (Contardo-Jara et al., 2011). The BCF of LNG decreases over time, as observed in our study, and the data are in accordance with fish plasma data (Fick et al., 2010). A previous study showed the elimination of LNG by degradation (Eckert et al., 2012), therefore a decreased BCF concentration suggest a regulation of the uptake. Also, in our study the uptake of LNG was much lower compared to the study by Fick and colleagues (Fick et al., 2010), but still higher than predicted by Huggett and colleagues (Huggett, 2003). In previous study Hugget and colleagues calculated a BCF of 0.46-fold for LNG in fish plasma (Huggett et al., 2003). This BCF based on the lipophilicity of LNG in human pharmacology and toxicology studies.

It is known that the reproduction of plants does not involve sex steroid hormones the way it does in vertebrates. Nevertheless, the biosynthetic pathways for the sterol precursors are ubiquitous and essential for cell membrane synthesis in all living cells (primarily stigma sterol in plants) (Taiz and Zeiger, 1998; Wynne-Edwards, 2001). It is noteworthy that we were able to detect for the first time a BCF for LNG in a similar range as reported for fish plasma on the submerged macrophyte *C. demersum*.

4.1.4 Metoprolol

So far, there are only a few studies dealing with physiological alterations in algae and invertebrates due to ß-blocker exposure. It was shown that the toxicity for algae was clearly higher than for Daphnia (Cleuvers, 2005). In this study where different ß-blockers were tested, Cleuvers described that MET has to be classified as toxic for aquatic organisms (Cleuvers and Ratte, 2002; Cleuvers, 2005). The green algae *Pseudokirchneriella subcapitata* indicated a substantial increase in non-specific toxicity with MET. The specific toxicity (inhibition of photosynthesis) towards

Pseudokirchneriella subcapitata remained unchanged (Radjenovic et al., 2011). Submerged macrophytes are constantly exposed to xenobiotics, which in general alters the stress status of organisms, and thereby provokes an imbalance in the homeostasis.

This study showed that MET was bioaccumulated in *C. demersum*. Accumulation occurred constantly from the first day at the concentration of 1.8 x 10^{-5} mol/l MET. In contrast for the 4.1 x 10^{-3} mol/l MET concentration a decrease of the BCF could be observed (43- to 17-fold), which may point to enhanced regulatory processes, e.g. metabolization. Furthermore, there is still uncertainty about the cellular uptake mechanisms of MET.

Dobson and Kell showed in a study a competition for binding sites taking place at a potential carrier itself, and hence an inhibition of influx (Dobson and Kell, 2008). Therefore, decreased BCF concentrations suggest a regulation of the uptake. If the uptake occurs passively by membrane permeation (Hayeshi et al., 2008), it can be assumed that there is no regulation at 1.8 x 10^{-5} mol/l MET.

For ß-blocker the toxicity classification can change from "not harmful" to "very toxic". This was shown in a further study with the unicellular algae *Desmodesmus subspicatus* when the substances were applied in single exposure or in a mixture (Escher et al., 2005; Hernando et al., 2004).

4.2 Metabolites of PhACs in *C. demersum*

The PhACs and xenobiotics are therefore stored as products of the detoxification pathway in the vacuole (Sandermann, 1992). In our study we found two metabolites of two different parental compounds (OH–IBU, Fig. 3.5 and *O*-desmethyl-MET, Fig. 3.6). None of the metabolites was found in the exposure medium, which also was routinely tested by LC-MS/MS.

OH–IBU is the major metabolite of IBU under toxic conditions (Zwiener et al., 2002). Carboxy-ibuprofen (CA-IBU), and carboxyhydratropic acid (CA-HA) could not be identified in biodegradation experiments. Inside the tissue of the macrophyte it seems to be that the biotransformation processes start rapidly after the uptake of the parental compound (Fig. 3.5). The rapid biotransformation of several microcystin variants in plants are described by Pflugmacher and colleagues (Pflugmacher et al., 2001).

The second analyzed metabolite was *O*–desmethyl–MET (Fig. 3.6). MET is mainly eliminated by oxidative metabolism (Belpaire et al., 1998). MET is detoxified via three different major

metabolites *O*-desmethyl-MET, MET acid, and hydroxyl-MET (Boralli et al., 2009; Liang et al., 2010; Ma et al., 2007). It has been recognized that conjugation is an important pathway of many compounds in microorganisms. A study with *Cunninghamella* showed the transformation of MET into different metabolites, one of these is *O*–desmethyl-MET (Ma et al., 2007).

4.3 Enzymatic response

For plants, survival under stress conditions is only possible if several antioxidant enzymes cooperate. Negative effects can be detected at different cellular levels, as altered metabolic rates of specific enzymes, as increased or decreased protein amounts or as changes in gene transcription. The presence of free radicals changes the activity of antioxidant enzymes in plants (Pflugmacher, 2004). This change in plant cells provides a defense system with a quick and balanced regeneration of active and reduced forms of antioxidants. Reactive oxygen species are constantly generated endogenously as a result of normal plant metabolism (Foyer et al., 1994), but also can be produced as by-products of biotransformation reactions of toxins or xenobiotics (Polle, 2001). Therefore, activities of antioxidant enzyme are often used to show the degree of injury in plants due to pollution.

Pharmaceuticals are not designed to exert acute toxic effects on non-target organisms. Their ongoing replenishment in the environment may lead to long term effects on aquatic organisms (Fent et al., 2006b). Antioxidant enzyme activities generally increase as a first response of plants suffering pollution.

We found different enzyme activity responses during the period of exposure and also in dependency to the concentrations. Our results confirm the importance of these antioxidant enzymes for the detoxification process of PhACs in *C. demersum*. Previous studies had shown enhanced oxidative stress response in *C. demersum* due to natural toxins (Esterhuizen-Londt et al., 2011; Pflugmacher and Sandermann, 1998; Pflugmacher, 2004; Pflugmacher et al., 1999). We cannot generally state oxidative stress due to an increase in ROS production with the PhACs we used for the exposure. Increasing ROS production was approved by POD activity of CBZ after 24h (Fig. 3.7B) and CAT activity of MET after 7d (Fig. 3.10A).

Furthermore, an important enzyme of the detoxification mechanisms is the GST activity (Pflugmacher and Sandermann, 1998; Pflugmacher et al., 1998). Decreased activities of the GST system can indicate a sign of saturation, whereas an increased activity is an indication of an

enhanced metabolism. Interestingly, our study confirms that GST responds differently to different PhACs exposures. Furthermore, it can be suggested that the tested PhACs, demonstrating a decreased GST activities are not detoxified through phase II metabolism. Therefore, GST cannot only be used as a biomarker for PhACs with this kind of question.

The significance or even the tendency towards inhabitation of oxidative stress enzymes caused a loss in conformational change in the protein leading to biological enzyme activity. Competitive inhibitory PhACs could resemble a closely related structure and molecular geometry for these enzymes. Therefore, they would compete for the same active site as the substrate molecule. However a competitive inhibition is usually reversible if sufficient substrate molecules are available to displace the inhibitor. Comparable results were described by Liao (Liao et al., 2005) with Cd^{2+}, Zn^{2+}, and acid rain; who showed decreased activities of antioxidant enzymes.

The inhibition of the enzyme is therefore dependent on the uptake concentration of PhACs by the organisms. *In vivo* an effect of PhACs is non-specific affecting metalloenzymes and non-metalloenzymes. This was shown in an *in vitro* study with BMAA, where the toxin was not specific and *in vitro* an effect might be due to a metabolic product, or a physiological response (Esterhuizen-Londt et al., 2011).

To protect the cellular function against oxidative stress, plant cells produce both antioxidant enzymes and nonenzymatic antioxidants such as glutathione and α–tocopherol. An important feature of these enzymes and nonenzymatic antioxidants is their inducibility under oxidative stress. Therefore, activities of antioxidant enzymes in plant cells are often used to show the degree of injury of plants due to pollution.

4.3.1 Carbamazepine

The pharmaceutical function of CBZ is to stabilize the inactivated state of voltage-gated sodium channels thus reducing their capacity for excitation. Plants are using other ion channel mechanisms (Hua et al., 2003), which could be the reason why *C. demersum* has shown a tendency to respond but did no show significant changes. Vernouillet and colleagues (Vernouillet et al., 2010) were able to show a 40 % increased activity of glutathion reductase with the algae *Pseudokirchneriella subcapitata*. The results of 10^{-5} mol CBZ concentration also revealed an increase of POD enzyme activity after 24 h.

Zhang and colleagues (Zhang et al., 2012) have shown a promotion of CAT activity in acute tests with two algae species (*S. obliquus* and *C. pyrenoidosa*) in a concentration range from 74 to

138 mg/l CBZ. The previously described results and our own ones (Fig. 3.7A), which were in a more environmentally plausible range than those of previous studies (Zhang et al., 2012), infer that CBZ caused some similar toxicity to aquatic non-target species. The differences were not significant due to the high standard deviation of the control.

4.3.2 Ibuprofen

As shown by the metabolite detection, *C. demersum* was able to form hydroxy-ibuprofen (OH-IBU). Therefore, the strong response of the enzyme activity could be associated with the detected metabolite OH-IBU. The inhibition or even the tendency toward inhibition, leads to the conclusion that IBU inhibits the enzyme activity of the antioxidant. In a study with paracetamol, the decreasing POD activity in *Triticum aestivum L.* roots after 14 days was described. This effect might be induced by H_2O_2 produced from the catalysis process of superoxide dismutase (An et al., 2009).

The detected effect of decreased sGST in our study was also observed in a study using freshwater cnidarian *Hydra attenuate* (Quinn et al., 2009). In a further study, it was shown that IBU has very weak photosynthesis effects on the marine algae *Fucus vesiculosus* (Oskarsson et al., 2012). This effect is of further interest to clarify the question, whether IBU also has toxicity effects on *C. demersum*.

4.3.3 Levonorgestrel

LNG is metabolized via the phase I over the CYP3A4 cytochrome complex in humans. We therefore expected an enhanced enzyme activity in the antioxidant response. LNG inhibits the enzyme activity of the antioxidant enzymes, and therefore it indirectly causes oxidative stress in cells. Pflugmacher (Pflugmacher et al., 2001) showed in a concerning microcystin study that the balance of reactive oxygen species in plants seems to be out of order after 72 h of exposure. Interestingly, the expected results of CAT response occurred during 24 h, but went only in the lower concentration down to normal till 7 d. The interesting case is that the used LNG concentration is in the relevant environmental range, comparing to the other PhACs we were used answering the question of a time-response effect of PhACs on the non-target organisms *C. demersum*.

4.3.4 Metoprolol

The strong CAT response of MET in *C. demersum* might be explained by the formation of *O*-desmethylation of MET. In a previous study, Cleuvers has shown the toxicity to the aquatic organism *Desmodesmus sp.* at a concentration of 7.9 mg/l MET and it was therefore classified in an OECD scheme (Cleuvers, 2003; OECD, 2001). In a further study with *Lemna minor*, an effect on growth rate test could not be shown (Cleuvers, 2005). In a study with 10^{-4} mol Propanol, a β-blocker, no effect was measured in the initial rise of fluorescence. This caused a decrease in the maximum chlorophyll fluorescence signal (Escher et al., 2005). This might be also an interesting case for *C. demersum*.

Interestingly, the response of 10^{-7} mol MET caused a significant drop in mGST activities (Fig. 3.10C) after 7d. This result is described in the literature as hormesis phenomenon. Hormesis has been documented in pharmacological and toxicological studies (Calabrese and Baldwin, 1998; Stebbing, 1982). The potential implications of this phenomenon are now being considered in terms of life-history traits and ecological risk assessment (Forbes, 2000).

Previous studies suggest that the enantiomers behave differently regarding biologically mediated environmental processes because biochemical receptors and enzymes distinguish chirality (Huang et al., 2012; Liu et al., 2005). In a pesticide study with the fresh water algae *Scenedesmus obliquus*, a previous study demonstrated the different toxic effects of the antioxidant enzymes (Huang et al., 2012). This difference occurs due to the racemate and enantiomers chiral compounds. Furthermore, it is also possible that similar targets govern different processes in different species (Seiler, 2002).

4.4 Chlorophyll pigments response

Changes in chlorophyll pigments have been frequently used as a sensitive physiological endpoint for the assessment of biological effects of substances coming into contact with plants (Brain et al., 2004; Cedergreen et al., 2007; Pflugmacher et al., 1999; Turgut and Fomin, 2002; Viet and Moser, 2003). Chloroplasts are especially subjected to oxidative stress during photosynthesis, and they have a multiplicity of protective mechanisms (glutathione among others) and repair systems. Significant decreases of photosynthetic oxygen production may have coincided with the possible breakdown of the detoxification enzymes. It may also be indicative of an acute stress condition in which a possible abrupt generation of high levels of free radicals can overload the antioxidant capacity and exert immediate effects.

Chlorophyll content meters are commonly used for stress measurement in plant including nitrogen-, sulfur-, xenobiotic- also pharmaceutically active compound induced stress. Concentration dependent inhibitory effects of chlorophyll synthesis of *C. demersum* can be observed in a sensitivity test using aquatic plants (Kamara and Pflugmacher, 2007b; Menone and Pflugmacher, 2005; Sersen et al., 1998). Former studies showed that the aromatic organic compound, 3-chlorobiphenyl, significantly inhibited the photosynthesis of *C. demersum* at a concentration of 5 mg/L (Menone and Pflugmacher, 2005). Further, the exposure to oak (*Quercus robur*) which is well known for its high tannin and phenolic contents (Salminen et al., 2004) suggests a possible interference with the electron transport chain from PSII to PSI in the chloroplast (site of photosynthesis) (Oettmeier et al., 1988; Pflugmacher, 2004).

The failure of the chlorophyll content and therefore of the photosynthetic system in *C. demersum* to recover after a relatively prolonged (one week) exposure to the PhACS may be attributed to its chemical composition. Previous studies demonstrated some plasticity in the photosynthetic systems when plants were exposed to other stress factors; increased $C0_2$ levels (Madsen et al., 1996), light intensity and water stress (Maxwell et al., 1994), temperature and inorganic carbon (Olesen and Madsen, 2000).

4.4.1 Carbamazepine

C. demersum was shown not to be vulnerable to CBZ by Zhang et al. (Zhang et al., 2012). Only a tendency towards a decrease of Chl a and increase of Chl b was noticeable after 7 days of exposure. In a previous study, the algae *S. capricornutum* showed a strong inhibition of Chl a, which was dependent on chloramphenicol (Banerji and Manmohan, 1966). A similar result was also obtained by Geoffroy et al. (Geoffroy et al., 2004), where Chl a content of *Selenastrum capricornutum* decreased significantly after 6 h exposure to the herbicide flumioxazin. It has been confirmed that concentration-dependent inhibitory effects for Chl a and some related ROS enzyme activity are reliable indicators of pollutant toxicity (Wang and Freemark, 1995).

4.4.2 Ibuprofen

It was known, that the anti-inflammatory drug IBU might cause indirect effects and followed by cascade effects combined with stress responses in the brown algae *Fucus vesiculosus* (Eriksson Wiklund et al., 2011), whereby IBU did not had such an effect as the simultaneously tested pharmaceutically active compound propanolol. Compared to the previous study by Eriksson Wiklund and colleagues (Eriksson Wiklund et al., 2011), IBU showed no concentration-

dependent effects on chlorophyll content of *C. demersum* during exposure. These effects could eventually have implications on community as well as serve as ecosystem benchmark.

With the knowledge that *C. demersum* is able to produce the metabolite OH-IBU, it is possible that this detoxification process protected the macrophyte from toxicity of this PhAC and therefore the photosynthesis is not compromised.

4.4.3 Levonorgestrel

The results indicated that *C. demersum* was vulnerable to LNG, which was shown on day 4 of exposure. The detected inhibition of Chl b could be shown after the exposure of day 4 (4 d). We are aware that this result is in contrast to previous studies analyzing stress effects on different chlorophyll contents in macrophytes (Pflugmacher, 2002; Pflugmacher et al., 2001; Weiss et al., 2000). To our knowledge there is no study available dealing with the stress effects of LNG on chlorophyll content of macrophytes or algae. The recovery of Chl b in *C. demersum* was apparent on the 7th day (7 d) of the exposure to LNG. Compared to CBZ, IBU, and MET; the concentration in the exposure medium of LNG was close to the known environmental surface water concentration in the low nanogram per liter range up to 30 ng/l (Viglino et al., 2008; Vulliet et al., 2007).

4.4.4 Metoprolol

The results indicated that PhACs (in this study: CBZ, IBU, LNG and MET) could influence chlorophyll pigments, whereby *C. demersum* showed a higher sensitivity to MET than to the other tested PhACs. This could be shown at both exposure endpoints; a reduction of the total chlorophyll pigment was shown at the higher concentration (4.1×10^{-3} mol/l MET). The results indicated that *C. demersum* was vulnerable to MET.

The decrease in primary production and chlorophyll content indicate that MET negatively affected photosynthesis, which is consistent with a study investigating the effect of another human pharmaceutical used against hypertension; propranolol on microalgae by Escher et al. (Escher et al., 2005). This could also be shown in a study with the brown macroalgae *F. vesiculosus* and the amphipod crustacean genus *Gammarus spp* (Oskarsson et al., 2012). The macroalgae was clearly affected by the exposure to propranolol in the experiments (Oskarsson et al., 2012). We hypothesize that the inhibitory effect of MET can be due to these pollutants, interfere with both, the synthesis of protochlorophyll and its subsequent conversion to chlorophyll.

Is there a structural relationship between PhACs and the measured inhibitory effect on plant photosynthetic oxygen production? MET contains stable free radicals of semi-quinone (Buszman and Rózańska, 2003). These radicals can be formed by spontaneous charge-transfer reactions through two single-electron steps between quinone and hydroquinone units (Sensi and Schnitzer, 1977). A further chemical reduction of quinoid structures leads to short-living free radicals (Sensi and Schnitzer, 1977). For several decades, quinones have been known to have inhibitory effects on the electron transfer chain in PS II (Oettmeier et al., 1988) and to act as Hill oxidants by absorbing electrons in the chloroplast (Trebst, 1972). Hence, it is most likely that the quinoid structures of some PhACs can act as electron scavengers and therefore inhibit the photosynthetic oxygen production of aquatic plants, this was also described by Pflugmacher and colleagues in a study with humic substances (Pflugmacher et al., 2006).

During the experiment it was noted that at a concentration of 4.1 x 10^{-3} mol/l MET the colour of the water changed, resulting in a cloudy medium. This was at first assumed to be released pigments, which could not be confirmed by comparison with the absorption spectrum. The coloration could, however, be a result of the loss of phenolic compounds, like phlorotannins, which can cause yellowish colorations in water (Schoenwaelder, 2002). Similar color changes in mediums used in algae exposure experiments have been observed in other studies, e.g., after exposure to copper (Mamboya et al., 2001) with a clear relationship to stress. The leakage of coloured substances from algae indicates that cell walls or membranes have been damaged by the exposure, which also affects photosynthesis. This is possibly due to MET being a selective *β*-blocker, which binds to specific receptor classes (Huggett, 2003), resulting in a malfunction of the following cascade and therefore has a further effect in the photosynthesis process.

5. Conclusions and perspectives

5.1 Conclusions

5.1.1 Bioaccumulation and biotransformation

The bioaccumulation of PhACs by organisms is a balance between passive uptake, bioaccumulation and depuration, and elimination of xenobiotic via biotransformation. The consideration of various physiological endpoints, such as biotransformation, antioxidant, cell protective processes or elimination, is not clear.

This study pointed out the exposure risks for aquatic organisms, like macrophytes were one option to enter over this way the contamination of food chain, which could possibly lead to "effect - accumulation".

- IBU and LNG showed an uptake in the non-target organism *C. demersum*.
- CBZ and MET were able to show incidences of bioaccumulation in the macrophyte.
- The formation of first step metabolites (OH-IBU and O-desmethyl MET) caused by *C. demersum*. The rates of metabolism of PhACs are generally significantly less than the uptake or bioaccumulation rate, resulting in marked PhACs bioaccumulation (Livingstone, 1998).
- PhACs affects the biotransformation and antioxidant related parameters at the one week exposure.

5.1.2 Effects

Little is known on the distribution of toxins in plants and signaling effects via reactive oxygen species, which might contribute to the understanding of bistability in lakes.

The non-target organism *C. demersum* exposed to PhACs showed diverse stress responses, from structural to biochemical changes. This provides a helpful tool to assess possible effects of PhACs on non-target organism. Non-enzymatic antioxidant could quench ROS directly or indirectly by regenerating ascorbic acid, another antioxidants compound (Foyer et al., 1994), which could be also tested.

- The acute study demonstrated the occurrence of physiological changes in the oxidative stress response of the submerged macrophyte due to CBZ, IBU, LNG, and MET exposure.
- *C. demersum* exposed to PhACs showed diverse stress responses, which could lead to structural or biochemical changes.
- The enzyme mGST was not strongly affected by low concentrations of the PhACs used.
- Furthermore, enzymes are located in different tissue organelles and also with different functions are inhibited in the same manner.

Even PhACs are water-soluble or with help of soluble compounds, this study proves that the accumulation dose in macrophytes showed enzymatic effects. Furthermore, it would be interesting to perform a transfer study of PhACs from the macrophytes to the next member in the food chain. The toxicity at environmental concentrations combined with possible bioaccumulation and/or biomagnification in food webs becomes of particular concern for higher levels.

5.1.3 Chlorophyll pigments

If in *C. demersum*, chlorophyll pigments were not affected, yet a significant photosynthetic inhibition occurred, suggesting that the mechanism of action was mainly that proposed by Pflugmacher and colleagues (Pflugmacher, 2004; Pflugmacher et al., 2007) involving the interruption of the electron transport chain. The results indicated that PhACs (CBZ, IBU, LNG and MET) could influence chlorophyll pigments, whereby *C. demersum* showed a higher sensitivity to MET than to the other PhACs tested.

- This could be shown at exposure endpoints; a reduction of Chl b and Chl total at the higher concentration (4.1 $x10^{-3}$mol/l MET) was detected.
- Only LNG was able to show incidences to inhibit chlorophyll pigments at the $1x10^{-8}$ mol/l LNG on the 4th day of exposure. But it recovered until 7th day.

5.2 Perspectives

The results of this thesis fill a gap of knowledge in the interaction between different PhACs and the macrophyte *C. demersum*. Nevertheless, this important ecological interface is highly variable and controlled by many factors; some of them are still not clear.

According to the results, it is suggested that further studies are needed to determine the mechanisms of accumulation, toxicity and stress resistance in aquatic plants upon exposure to PhACs.

The ability of macrophytes to metabolize pharmaceuticals does not only help in refining bioaccumulation factor prediction, could also help to further improve the overall risk assessment of PHACs. In the future more specialized ecological studies would enable the distinction of species with particular accumulative capacities to one or several pharmaceuticals, thereby serving as bio-indicator of contaminated areas.

The detected values of PhACs in the environment are below the levels predicted to cause harm to humans, but if they are below the level to cause acute or even chronic toxicity to non–target organisms needs to be clarified. Also an investigation would be interesting to test the possibility to form stable bound residues with cell wall components. Bound residues might have also influence on the food web, if the toxin is released again. The toxicity at environmental concentrations combined with possible bioaccumulation and/or biomagnification in food webs becomes of particular concern for higher levels of the food chains.

Further work should address the following question; how do macrophytes react under survival stress conditions if several antioxidants cooperate together with antioxidant enzymes, providing a good defense system. Also changes in gene expression suggest a cellular effect of PhACs on *C. demersum*. It is mandatory to examine the long - term effects of PhACs exposure in the aquatic macrophyte *C. demersum*. This could also impact growth and reproduction.

The enzyme assay results are shown a different manifestation of the antioxidant stress response. It can be suggested to become used for accurate site assessment either with enzymatic or molecular biomarkers. Each other with a specific suite complementing and the expected pollutants, getting there with an integrated view of the pollution scenario and its biological effects. One compound alone may prove little effects, but when acting together with other compounds may pose a hazard, which may be underestimated by focusing on individual compounds alone (Fent et al., 2006b).

Further knowledge and tools derived from the *C. demersum* genome in combination with studies in the laboratory and field should bring new light into this subject. In view of the ongoing rapid changes in environment because of anthropogenic and climate changes, several factors (CO_2 and

nitrogen compounds) are highlighted and their impact in single or in combination exposures should be evaluated.

The excretions of unchanged forms of PhACs, along with the inadequacy of WWTPs removal methods constitute the major pathways of entrance into the environment. Therefore, there is a need to find viable alternatives to underline the now implemented methods to improve the removal efficiencies of WWTPs. It is of great relevance to develop strategies of remediation of habitats in order to decrease the impact of PhACs.

6. Literature Cited

An, J., Zhou, Q., Sun, F., Zhang, L., 2009. Ecotoxicological effects of paracetamol on seed germination and seedling development of wheat (Triticum aestivum L.). J. Hazard. Mater. 169, 751–7.

Ankley, G.T., Brooks, B.W., Huggett, D.B., Sumpter, J.P., 2007. Repeating History : Science (80).

Anzenbacher, P., Anzenbacherova, E., 2001. Cytochromes P450 and metabolism of. Cell Mol Life Sci 58.

Aravind, P., Prasad, M.N.V., 2005. Modulation of cadmium-induced oxidative stress in Ceratophyllum demersum by zinc involves ascorbate-glutathione cycle and glutathione metabolism. Plant Physiol. Biochem. 43, 107–16.

Asada, K., 1992. Ascorbate peroxidase - a hydrogen peroxide-scavenging enzyme in plants. Physiol. Plant. 85, 235–41.

Asada, K., 2006. Ascorbate peroxidase - a hydrogen peroxide scavenging enzyme in plants.pdf. Physiol. Plant. 85, 235–41.

Baier, M., Dietz, K.-J., 2005. Chloroplasts as source and target of cellular redox regulation: a discussion on chloroplast redox signals in the context of plant physiology. J. Exp. Bot. 56, 1449–62.

Banerji, D., Manmohan, M.., 1966. Chlorophyll formation in isolated pumpkin cotyledons in the presence of kinetin and chloramphenicol. Oxford Journals 8, 263 – 68.

Baudhuin, U., Karpinski, S., Mullineaux, P.M., 1964. Tissue Fractionation Studies XVII. Omtracellular distribution of monoamine oxidase, aspartate amino-transferase, d-amino acid oxidase and catalase in rat liver tissue. Biochem. J. 92, 179–84.

Bayne, B.L., Moore, M.N., Widdows, J., Livingstone, D.R., Salkeld, P., 1979. Measurement of the responses of individuals to environmental stress and pollution: studies with bivalve molluscs. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 286, 563–81.

Bechtold, U., Karpinski, S., Mullineaux, P.M., 2005. The influence of the light environment and photosynthesis on oxidative signalling responses in plant-biotrophic pathogen interactions. Plant, Cell Environ. 28, 1046–55.

Behera, S.K., Oh, S.Y., Park, H.S., 2012. Sorptive removal of ibuprofen from water using selected soil minerals and activated carbon. Int. J. Environ. Sci. Technol. 9, 85–94.

Belpaire, F.M., Wijnant, P., Temmerman, a, Rasmussen, B.B., Brøsen, K., 1998. The oxidative metabolism of metoprolol in human liver microsomes: inhibition by the selective serotonin reuptake inhibitors. Eur. J. Clin. Pharmacol. 54, 261–4.

Bendz, D., Paxéus, N. a, Ginn, T.R., Loge, F.J., 2005. Occurrence and fate of pharmaceutically active compounds in the environment, a case study: Höje River in Sweden. J. Hazard. Mater. 122, 195–204.

Bergmeyer, H.U. (ed. ., n.d. Methods of enzymatic analysis., Vol. I-XII. ed. Weinheim.

Besse, J.-P., Garric, J., 2009. Progestagens for human use, exposure and hazard assessment for the aquatic environment. Environ. Pollut. 157, 3485–94.

Best, E., Visser, H., 1987. growth of the submerged macrophyte Ceratophyllum demersum L. in mesotrophic Lake Vechten in relation to insolation, temperature and reserve carbohydrates. Hydrobiologia 243, 231–43.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–7.

Blokhina, O., 2003. Antioxidants, Oxidative Damage and Oxygen Deprivation Stress: a Review. Ann. Bot. 91, 179–94.

Boralli, V.B., Coelho, E.B., Lanchote, V.L., 2009. Influence of Quinidine , Cimetidine , and Ketoconazole on the Enantioselective Pharmacokinetics and Metabolism of Metoprolol in Rats. Chirality 8, 886–93.

Borg, K.O., Carlsson, E., Hoffmann, K., Jonsson, T., Thorin, H., 1975. by C " 3 CH2- CH2- OCH3 125–35.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–54.

Brain, R. a, Johnson, D.J., Richards, S.M., Hanson, M.L., Sanderson, H., Lam, M.W., Young, C., Mabury, S. a, Sibley, P.K., Solomon, K.R., 2004. Microcosm evaluation of the effects of an eight pharmaceutical mixture to the aquatic macrophytes Lemna gibba and Myriophyllum sibiricum. Aquat. Toxicol. 70, 23–40.

Breton, H., Cociglio, M., Bressolle, F., Peyriere, H., Blayac, J.P., Hillaire-Buys, D., 2005. Liquid chromatography-electrospray mass spectrometry determination of carbamazepine, oxcarbazepine and eight of their metabolites in human plasma. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 828, 80–90.

Brun, G.L., Bernier, M., Losier, R., Doe, K., Jackman, P., Lee, H.-B., 2006. Pharmaceutically active compounds in atlantic canadian sewage treatment plant effluents and receiving waters, and potential for environmental effects as measured by acute and chronic aquatic toxicity. Environ. Toxicol. Chem. 25, 2163–76.

Buser, H., Mu, M.D., Wa, C.-, 1999. Occurrence and Environmental Behavior of the Chiral Pharmaceutical Drug Ibuprofen in Surface Waters and in Wastewater. Environ. Sci. Technol. 2529–35.

Buszman, E., Rózańska, R., 2003. Interaction of quinidine, disopyramide and metoprolol with melanin in vitro in relation to drug-induced ocular toxicity. Pharmazie 58, 507–11.

Calabrese, E.J., Baldwin, L. a, 1998. Hormesis as a biological hypothesis. Environ. Health Perspect. 106 Suppl , 357–62.

Carballa, M., Omil, F., Lema, J.M., Llompart, M., García-Jares, C., Rodríguez, I., Gómez, M., Ternes, T., 2004. Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. Water Res. 38, 2918–26.

Castillo, F.J., Miller, P.., Greppin, H., 1987. Extracellular biochemical markers of photochemical oxidant air pollutant damage to Norway spruce. Experimentia 43, 111–5.

Cedergreen, N., Abbaspoor, M., Sørensen, H., Streibig, J.C., 2007. Is mixture toxicity measured on a biomarker indicative of what happens on a population level? A study with Lemna minor. Ecotoxicol. Environ. Saf. 67, 323–32.

Chen, X., Pauly, U., Rehfus, S., Bester, K., 2009. Personal care compounds in a reed bed sludge treatment system. Chemosphere 76, 1094–101.

Christen, V., Hickmann, S., Rechenberg, B., Fent, K., 2010. Highly active human pharmaceuticals in aquatic systems : A concept for their identification based on their mode of action . 96, 2010.

Claiborne, B.J., Amaral, D.G., Cowan, W.M., 1986. A light and electron microscopic analysis of the mossy fibers of the rat dentate gyrus. J. Comp. Neurol. 246, 435–58.

Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol. Lett. 142, 185–94.

Cleuvers, M., 2005. Initial risk assessment for three beta-blockers found in the aquatic environment. Chemosphere 59, 199–205.

Cleuvers, M., Ratte, H.-T., 2002. Phytotoxicity of coloured substances: is Lemna duckweed an alternative to the algal growth inhibition test? Chemosphere 49, 9–15.

Coleman, J.O. Do, Blake-kajff, M.M., 1997. Detoxification of xenobiotics by plants : chemical modification and vacuolar compartmentation. Science (80). 1385.

Contardo-Jara, V., Lorenz, C., Pflugmacher, S., Nützmann, G., Kloas, W., Wiegand, C., 2011. Molecular effects and bioaccumulation of levonorgestrel in the non-target organism Dreissena polymorpha. Environ. Pollut. 159, 38–44.

Contardo-Jara, V., Pflugmacher, S., Nützmann, G., Kloas, W., Wiegand, C., 2010. The beta-receptor blocker metoprolol alters detoxification processes in the non-target organism Dreissena polymorpha. Environ. Pollut. 158, 2059–66.

Crane, M., Watts, C., Boucard, T., 2006. Chronic aquatic environmental risks from exposure to human pharmaceuticals. Sci. Total Environ. 367, 23–41.

Dat, J., Vandenabeele, S., Vranová, E., Van Montagu, M., Inzé, D., Van Breusegem, F., 2000. Dual action of the active oxygen species during plant stress responses. Cell. Mol. Life Sci. 57, 779–95.

Daughton, C.G., Ternes, T. a, 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ. Health Perspect. 107 Suppl , 907–38.

De Alda, M.J.L., Gil, A., Paz, E., Barcelo, D., 2002. Occurrence and analysis of estrogens and progestogens in river sediments by liquid. Analyst 127, 1299–304.

Delépée, R., Pouliquen, H., Le Bris, H., 2004. The bryophyte Fontinalis antipyretica Hedw. bioaccumulates oxytetracycline, flumequine and oxolinic acid in the freshwater environment. Sci. Total Environ. 322, 243–53.

Denisov, E.T., Afanas´ev, I.B., 2005. Oxidation and Antioxidants. byTaylor & Friends Group. Boca Raton FL 33487- 2742.

Dietz, K.-J., Pfannschmidt, T., 2011. Novel regulators in photosynthetic redox control of plant metabolism and gene expression. Plant Physiol. 155, 1477–85.

Dobson, P.D., Kell, D.B., 2008. Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? Nat. Rev. Drug Discov. 7, 205–20.

Doust, J.L., Schmidt, M., Doust, L.L., 1994. Biological assessment of aquatic pollution: a review, with emphasis on plants as biomonitors. Biol. Rev. Camb. Philos. Soc. 69, 147–86.

Dröge, W., 2002. Free radicals in the physiological control of cell function. Physiol. Rev. 82, 47–95.

Drotar, A., Phelps, P., Fall, R., 1985. Evidence for glutathione peroxidase activities in cultured plant cells. Plant Sci. 42, 35–40.

Eckert, V., Bensmann, H., Zegenhagen, F., Weckenmann, J., Sörensen, M., 2012. Elimination of Hormones in Pharmaceutical Waste Water. pharmind 3, 487–92.

Ericson, H., Thorsén, G., Kumblad, L., 2010. Physiological effects of diclofenac, ibuprofen and propranolol on Baltic Sea blue mussels. Aquat. Toxicol. 99, 223–31.

Eriksson Wiklund, A., Oskarsson, H., Thorsén, G., Krumblad, L., 2011. Behavioural and physiological responses to - pharmaceutical exposure in macroalgae and grazers from a Baltic Sea littoral community. Aquat. Biol. 14, 29–39.

Escher, B.I., Bramaz, N., Eggen, R.I.L., Richter, M., 2005. In vitro assessment of modes of toxic action of pharmaceuticals in aquatic life. Environ. Sci. Technol. 39, 3090–100.

Esterhuizen, M., Pflugmacher, S., Downing, T.G., 2011. β-N-Methylamino-L-alanine (BMAA) uptake by the aquatic macrophyte Ceratophyllum demersum. Ecotoxicol. Environ. Saf. 74, 74–7.

Esterhuizen-Londt, M., Pflugmacher, S., Downing, T.G., 2011. The effect of β-N-methylamino-L-alanine (BMAA) on oxidative stress response enzymes of the macrophyte Ceratophyllum demersum. Toxicon 57, 803–10.

Fent, K., Escher, C., Caminada, D., 2006a. Estrogenic activity of pharmaceuticals and pharmaceutical mixtures in a yeast reporter gene system. Reprod. Toxicol. 22, 175–85.

Fent, K., Weston, A. a, Caminada, D., 2006b. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76, 122–59.

Ferrari, B., Paxéus, N., Lo Giudice, R., Pollio, A., Garric, J., 2003. Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. Ecotoxicol. Environ. Saf. 55, 359–70.

Fick, J., Rh, L., Parkkonen, J., Arvidsson, B., Tysklind, M., Dg, L., 2010. Therapeutic levels of levonorgestrel detected in blood plasma of fish : results from screening rainbow trout exposed to treated sewage effluents . 44, 20222725.

FitzGerald, G. a, Patrono, C., 2001. The coxibs, selective inhibitors of cyclooxygenase-2. N. Engl. J. Med. 345, 433–4

Forbes, V.E., 2000. Is hormesis an evolutionary expection? Funct. Ecol. 14, 12–14.

Foureman, G.L., Harris, C., Guengerich, F.P., Bend, J.R., 1989. Stereoselectivity of styrene oxidation in microsomes and in purified cytochrome P-450 enzymes from rat liver. J. Pharmacol. Exp. Ther. 248, 492–7.

Foyer, C.H., Lelandais, M., Kunert, K.J., 1994. Photooxidative stress in plants. Physiol. Plant. 92, 696–717.

Foyer, C.H., Noctor, G., 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell 17, 1866–75.

Fridovich, I., 1998. Oxygen toxicity: a radical explanation. J. Exp. Biol. 201, 1203–9.

Fridovich, I., 2004. Mitochondria : are they the seat of senescence ? Aging Cell 13–16.

Gagné, F., Blaise, C., Fournier, M., Hansen, P.D., 2006. Effects of selected pharmaceutical products on phagocytic activity in Elliptio complanata mussels. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 143, 179–86.

Geoffroy, L., Frankart, C., Eullaffroy, P., 2004. Comparison of different physiological parameter responses in Lemna minor and Scenedesmus obliquus exposed to herbicide flumioxazin. Environ. Pollut. 131, 233–41.

Glatt, H., 2000. Sulfotransferases in the bioactivation of. Chem. Biol. Interact. 129, 2000.

González-Naranjo, V., Boltes, K., 2013. Toxicity of ibuprofen and perfluorooctanoic acid for risk assessment of mixtures in aquatic and terrestrial environments. Int. J. Environ. Sci. Technol. 11, 1743–1750.

Grillo, M.P., Hua, F., 2008. Enantioselective Fromation of Ibuprofen-S-Acyl-Gluthione in Virto in Incubations of Ibuprofen with Rat Hepatocytes. Chem. Res. Toxicol. 21, 1749–59.

Gros, M., Petrović, M., Barceló, D., 2006. Development of a multi-residue analytical methodology based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for screening and trace level determination of pharmaceuticals in surface and wastewaters. Talanta 70, 678–90.

Guissé, B., Srivastava, A., Strasser, R.J., 1995. The polyphasic rise of the chlorophyll a fluorescence (O-K-J-I-P) in heat stressed leaves. Arch. Sci. Genève 48, 147–160.

Habig, W., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferase: the first step in mercapturic acid formation. J. Biol. Chem. 249, 7130–9.

Harms, H.H., 1992. In-vitro Systems for Studying Phytotoxicity and Metabolic Fate of Pesticides and Xenobiotics in Plants *. Cell 277–81.

Hausladen, A., Alscher, R., 1993. Gluthathione. In. CRC Press, CRC Press, Boca Ra-Ton, FL.

Hayes, J.D., Flanagan, J.U., Jowsey, I.R., 2005. Glutathione transferases. Annu. Rev. Pharmacol. Toxicol. 45, 51–88.

Hayes, J.D., Pulford, D.J., 1995. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit. Rev. Biochem. Mol. Biol. 30, 445–600.

Hayeshi, R., Masimirembwa, C., Mukanganyama, S., Ungell, A.L., 2008. Lysosomal trapping of amodiaquine : impact on transport across intestinal epithelia models . Biopharm Drug Dispos. 29, 18570280.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol. Lett. 131, 5–17.

Heckmann, L.-H., Callaghan, A., Hooper, H.L., Connon, R., Hutchinson, T.H., Maund, S.J., Sibly, R.M., 2007. Chronic toxicity of ibuprofen to Daphnia magna: Effects on life history traits and population dynamics. Toxicol. Lett. 172, 137–45.

Hernando, M., Petrovic, M., Fernandezalba, a, Barcelo, D., 2004. Analysis by liquid chromatography?electrospray ionization tandem mass spectrometry and acute toxicity evaluation for ?-blockers and lipid-regulating agents in wastewater samples. J. Chromatogr. A 1046, 133–40.

Hua, B., Mercier, R.W., Leng, Q., Berkowitz, G.A., 2003. Plants Do It Differently . A New Basis for Potassium / Sodium Selectivity in the Pore of an Ion Channel 1 132, 1353–61.

Huang, L., Lu, D., Diao, J., Zhou, Z., 2012. Enantioselective toxic effects and biodegradation of benalaxyl in Scenedesmus obliquus. Chemosphere 87, 7–11.

Huggett, D.B., 2003. Comparison of in Vitro and in Vivo Bioassays for Estrogenicity in Effluent from North American Municipal Wastewater Facilities. Toxicol. Sci. 72, 77–83.

Huggett, D.B., Cook, J.C., Ericson, J.F., Williams, R.T., 2003. A theoretical model for utilizing mammalian pharmacology and saftey data to prioritize potential impacts of human pharamceuticals to fish. Hum. Ecol. Risk Assess. 9, 1789 – 99.

Imlay, J. a, Linn, S., 1988. DNA damage and oxygen radical toxicity. Science 240, 1302–9.

Inskeep, W.P., Bloom, P.R., 1985. Extinction Coefficients of Chlorophyll a and b in 483–5.

Jones, H.S., Trollope, H.T., Hutchinson, T.H., Panter, G.H., Chipman, J.K., 2012. Metabolism of ibuprofen in zebrafish larvae. Xenobiotica.

Jones, O. a H., Voulvoulis, N., Lester, J.N., 2002. Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. Water Res. 36, 5013–22.

Kamara, S., Pflugmacher, S., 2007a. Phragmites australis and Quercus robur leaf extracts affect antioxidative system and photosynthesis of Ceratophyllum demersum. Ecotoxicol. Environ. Saf. 67, 240–6.

Kamara, S., Pflugmacher, S., 2007b. Acclimation of Ceratophyllum demersum to stress imposed by Phragmites australis and Quercus robur leaf extracts. Ecotoxicol. Environ. Saf. 68, 335–42.

Kim, H., Jeong, K., Lee, S., Jung, S., 2003. Molecular Modeling of the Chiral Recognition of Propranolol Enantiomers by a β -Cyclodextrin 24, 95–8.

King, R.S., Teitel, C.H., Kadlubar, F.F., 2000. In vitro bioactivation of N -hydroxy-2-amino- α -carboline ence of GSH . These results suggest that bioactivation of N-OH-A α C may be a significant source of DNA damage in human tissues after dietary exposure to A α C and that the relative contribution o 21, 1347–54.

Kobbia, I. a, Battah, M.G., Shabana, E.F., Eladel, H.M., 2001. Chlorophyll a fluorescence and photosynthetic activity as tools for the evaluation of simazine toxicity to Protosiphon botryoides and Anabaena variabilis. Ecotoxicol. Environ. Saf. 49, 101–5.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–11.

Kruger, G.H.J., Tsimilli-Michael, M., Strasser, R.J., 1997. Light stress provokes plastic and elastic modifications in structure and function of photosystem II in camellia leaves. Physiol. Plant. 101, 265–77.

Kuch, H.M., Ballschmiter, K., 2000. Determination of endogenous and exogenous estrogens in effluents from sewage treatment plants at the ng/L-level. Fresenius. J. Anal. Chem. 366, 392–5.

Kümmerer, K., 2009. The presence of pharmaceuticals in the environment due to human use--present knowledge and future challenges. J. Environ. Manage. 90, 2354–66.

Kunkel, U., Radke, M., 2011. Reactive tracer test to evaluate the fate of pharmaceuticals in rivers. Environ. Sci. Technol. 45, 6296–302.

Lajeunesse, A., Vernouillet, G., Eullaffroy, P., Gagnon, C., Juneau, P., Sauvé, S., 2009. Determination of carbamazepine in aquatic organisms by liquid-liquid extraction and liquid chromatography-tandem mass spectrometry. J. Environ. Monit. 11, 723–5.

Langenhoff, A., Inderfurth, N., Veuskens, T., Schraa, G., Blokland, M., Kujawa-Roeleveld, K., Rijnaarts, H., 2013. Microbial removal of the pharmaceutical compounds Ibuprofen and diclofenac from wastewater. Biomed Res. Int. 1–9.

Liang, X., Jiang, H., Xi, C., 2010. Human DBS sampling with LC-MS/MS for enantioselective determination of metoprolol and its metabolite *O*-desmethyl metoprolol. Bioanalysis 2 (8), 1437–48.

Liao, B., Liu, H., Zeng, Q., Yu, P., Probst, A., Probst, J.-L., 2005. Complex toxic effects of Cd2+, Zn2+, and acid rain on growth of kidney bean (Phaseolus vulgaris L). Environ. Int. 31, 891–5.

Lishman, L., Smyth, S.A., Sarafin, K., Kleywegt, S., Toito, J., Peart, T., Lee, B., Servos, M., Beland, M., Seto, P., 2006. Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. Sci. Total Environ. 367, 544–58.

Liu, W., Gan, J., Schlenk, D., Jury, W. a, 2005. Enantioselectivity in environmental safety of current chiral insecticides. Proc. Natl. Acad. Sci. U. S. A. 102, 701–6.

Livingstone, D.R., 1998. The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 120, 43–9.

Lorenz, C., Contardo-Jara, V., Pflugmacher, S., Wiegand, C., Nützmann, G., Lutz, I., Kloas, W., 2011. The synthetic gestagen levonorgestrel impairs metamorphosis in Xenopus laevis by disruption of the thyroid system. Toxicol. Sci. 123, 94–102.

Ma, B., Huang, H., Chen, X., Sun, Y., Lin, L., Zhong, D., 2007. Biotransformation of metoprolol by the fungus Cunninghamella blakesleeana. Acta Pharmacol. Sin. 28, 1067–74.

Madsen, T.., Maberly, S.., Bowes, G., 1996. Photosynthetic acclimation of submersed angiosperms to C0 and HCO3. Aquat. Bot. 53, 15 –30.

Mamboya, F.A., Pratap, H.B., Mtolera, M., Björk, M., 2001. The effect of copper on the daily growth rate and photosynthetic efficiency of the brown macroalga Padina boergesenii, Publ. IMS/WIOMSA. Marine Science Development in Tanzania and Eastern Africa. Proceedings of the 20th Anniversary Conference on Advances in Marine Science in Tanzania, 28th Junee1st July 1999, Zanzibar, Tanzania.

Matamoros, V., Duhec, A., Albaigés, J., Bayona, J.M., 2008. Photodegradation of Carbamazepine, Ibuprofen, Ketoprofen and 17α-Ethinylestradiol in Fresh and Seawater. Water. Air. Soil Pollut. 196, 161–8.

Maxwell, C., Griffiths, H., Young, A.J., 1994. Photosynthetic acclimation to light regime and water stress by the C3-CAM epiphyte Guzmania monostachia: Gas-exchange characteristiecs, photochemical efficiency and the xanthophyll cycle. Funct. Ecol. 8, 746 – 54.

Menone, M.L., Pflugmacher, S., 2005. Effects of 3-chlorobiphenyl on photosynthetic oxygen production, glutathione content and detoxication enzymes in the aquatic macrophyte Ceratophyllum demersum. Chemosphere 60, 79–84.

Metcalf, J.S., Beattie, K. a, Pflugmacher, S., Codd, G. a, 2000. Immuno-crossreactivity and toxicity assessment of conjugation products of the cyanobacterial toxin, microcystin-LR. FEMS Microbiol. Lett. 189, 155–8.

Mishra, S., Srivastava, S., Tripathi, R.D., Dwivedi, S., Shukla, M.K., 2008. Response of Antioxidant Enzymes in Coontail (Ceratophyllum demersum L .) Plants Under Cadmium Stress. Environ. Toxicol. 294–301.

Mitsou, K., Koulianou, A., Lambropoulou, D., Pappas, P., Albanis, T., Lekka, M., 2006. Growth rate effects, responses of antioxidant enzymes and metabolic fate of the herbicide Propanil in the aquatic plant Lemna minor. Chemosphere 62, 275–84.

Mittova, V., Theodoulou, F.L., Kiddle, G., Volokita, M., Tal, M., Foyer, C.H., Guy, M., 2004. Comparison of mitochondrial ascorbate peroxidase in the cultivated tomato , Lycopersicon esculentum , and its wild , salt-tolerant relative , L . pennellii – a role for matrix isoforms in protection against oxidative damage 237–50.

Mohr, S., Schröder, H., Feibicke, M., Berghahn, R., Arp, W., Nicklisch, a, 2008. Long-term effects of the antifouling booster biocide Irgarol 1051 on periphyton, plankton and ecosystem function in freshwater pond mesocosms. Aquat. Toxicol. 90, 109–20.

Nebbia, C., Ceppa, L., Dacasto, M., Nachtmann, C., Carletti, M., 2001. Oxidative monensin metabolism and cytochrome P450 3A content and functions in liver microsomes from horses, pigs, broiler chicks, cattle and rats. J. Vet. Pharmacol. Ther. 24, 399–403.

Nebert, D.W., Dalton, T.P., 2006. The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. Nat. Rev. Cancer 6, 947–60.

Nelson, D., Cox, M., 2005. Lehninger priciples of biochemsitry, 4th ed. New York, NY.

Nimptsch, J., Pflugmacher, S., 2005. Substrate specificities of cytosolic glutathione-S transferases in five different species of the aqutic macrophyte Myriophyllum. J. Appl. Bot. Food Qual. - Angew. Bot. 79, 94–9.

Nimptsch, J., Pflugmacher, S., 2007. Ammonia triggers the promotion of oxidative stress in the aquatic macrophyte Myriophyllum mattogrossense. Chemosphere 66, 708–14.

Nimptsch, J., Wiegand, C., Pflugmacher, S., 2008. Cyanobacterial toxin elimination via bioaccumulation of MC-LR in aquatic macrophytes: an application of the "Green Liver Concept". Environ. Sci. Technol. 42, 8552–7.

OECD, O. for E. co-operation and D., 2001. Highlights of the OECD Environmental Outlook, Organisation for Economic co-operation and development. OECD Publication, Paris, France.

Oettmeier, W., Masson, K., Donner, A., 1988. Anthraquinone inhibitors of photosystem II electron transport. FEBS Lett. 259–62.

Olesen, B., Madsen, T.., 2000. Growth and physiological acclimation to temperature and inorganic carbon availability to two submerged aquatic macrophyte species, Callitriche cophocarpa and Elodea canadensis. Funct. Ecol. 14, 252–60.

Ollers, S., Singer, H.P., Fässler, P., Müller, S.R., 2001. Simultaneous quantification of neutral and acidic pharmaceuticals and pesticides at the low-ng/l level in surface and waste water. J. Chromatogr. A 911, 225–34.

Oskarsson, H., Eriksson Wiklund, A.-K., Lindh, K., Kumblad, L., 2012. Effect studies of human pharmaceuticals on Fucus vesiculosus and Gammarus spp. Mar. Environ. Res. 74, 1–8.

Owen, S.F., Giltrow, E., Huggett, D.B., Hutchinson, T.H., Saye, J., Winter, M.J., Sumpter, J.P., 2007. Comparative physiology, pharmacology and toxicology of beta-blockers: mammals versus fish. Aquat. Toxicol. 82, 145–62.

Palmgrén, J.J., Mönkkönen, J., Korjamo, T., Hassinen, A., Auriola, S., 2006. Drug adsorption to plastic containers and retention of drugs in cultured cells under in vitro conditions. Eur. J. Pharm. Biopharm. 64, 369–78.

Peñuelas, J., Llusià, J., Asensio, D., 2005. Linking isoprene with plant thermotolerance , antioxidants and monoterpene emissions 278–86.

Pfannschmidt, T., 2003. Chloroplast redox signals: how photosynthesis controls its own genes. Trends Plant Sci. 8, 33–41.

Pflugmacher, S., 2002. Possible allelopathic effects of cyanotoxins, with reference to microcystin-LR, in aquatic ecosystems. Environ. Toxicol. 17, 407–13.

Pflugmacher, S., 2004. Promotion of oxidative stress in the aquatic macrophyte Ceratophyllum demersum during biotransformation of the cyanobacterial toxin microcystin-LR. Aquat. Toxicol. 70, 169–78.

Pflugmacher, S., Codd, G.A., Steinberg, C.E.W., 1998. Effects of the Cyanobacterial Toxin Microcystin-LR on Detoxication Enzymes in Aquatic Plants. Environ. Toxicol. 111–5.

Pflugmacher, S., Olin, M., Kankaanpää, H., 2007. Nodularin induces oxidative stress in the Baltic Sea brown alga Fucus vesiculosus (Phaeophyceae). Mar. Environ. Res. 64, 149–59.

Pflugmacher, S., Pietsch, C., Reiger, W., Paul, A., Preuer, T., Zwirnmann, E., 1999. Humic substances and their direct effects on the physiology of aquatic plants, Humic subs. ed. Taylor and Francis, New York, NY.

Pflugmacher, S., Pietsch, C., Reiger, W., Paul, A., Preuer, T., Zwirnmann, E., Steinberg, C.E.W., 2003. Humic substance and their direct effects on the physiology of aquatic plants., Humic subs. ed. Taylor and Francis, New York, NY.

Pflugmacher, S., Pietsch, C., Rieger, W., Steinberg, C.E.W., 2006. Dissolved natural organic matter (NOM) impacts photosynthetic oxygen production and electron transport in coontail Ceratophyllum demersum. Sci. Total Environ. 357, 169–75.

Pflugmacher, S., Sandermann, H., 1998. Cytochrome P450 monooxygenases for fatty acids and xenobiotics in marine macroalgae. Plant Physiol. 117, 123–8.

Pflugmacher, S., Steinberg, C.E.W., 1997. Activity of phase I and phase II detoxication enzymes in aquatic macrophytes 71, 1997.

Pflugmacher, S., Wiegand, C., Beattie, K. a, Krause, E., Steinberg, C.E., Codd, G. a, 2001. Uptake, effects, and metabolism of cyanobacterial toxins in the emergent reed plant Phragmites australis (cav.) trin. ex steud. Environ. Toxicol. Chem. 20, 846–52.

Polle, A., 2001. Dissecting the superoxide dismutase-ascorbate-glutathione-pathway in chloroplasts by metabolic modeling. Computer simulations as a step towards flux analysis. Plant Physiol. 126, 445–62.

Pomati, F., Orlandi, C., Clerici, M., Luciani, F., Zuccato, E., 2008. Effects and interactions in an environmentally relevant mixture of pharmaceuticals. Toxicol. Sci. 102, 129–37.

Quinn, B., Gagné, F., Blaise, C., 2009. Evaluation of the acute, chronic and teratogenic effects of a mixture of eleven pharmaceuticals on the cnidarian, Hydra attenuata. Sci. Total Environ. 407, 1072–9.

Quzounidou, G., Moustakas, M., Strasser, R.J., 1997. Sites of action of copper in the photosynthetic apparatus of maize leaves: Kinetic analysis of chlorophyll fluorescence, oxygen evolution, absorption changes and thermal dissipation as monitored by photoacoustic signals. Aust. J. Plant Physiol. 24, 81–90.

Radjenovic, J., Escher, B.I., Rabaey, K., 2011. Electrochemical degradation of the β-blocker metoprolol by Ti/Ru 0.7 Ir 0.3 O 2 and Ti/SnO 2-Sb electrodes. Water Res. 45, 3205–14.

Ribeyre, F., Boudou, a, 1994. Experimental study of inorganic and methylmercury bioaccumulation by four species of freshwater rooted macrophytes from water and sediment contamination sources. Ecotoxicol. Environ. Saf. 28, 270–86.

Routledge, E.J., Sheahan, D., Desbrow, C., Brighty, G.C., Waldock, M., Sumpter, J.P., 1998. Identification of Estrogenic Chemicals in STW Effluent . 2 . In Vivo Responses in Trout and Roach 32, 1559–65.

Salminen, J., Roslin, T., Karonen, M., Sinkkonen, J., Pihlaka, K., Pulkkinen, P., 2004. Seasonal variation in the content of hydrolizable tannins, flavonoid glycosides and proanthocyanidins in oak leaves. J. Chem. Ecol. 30, 1693–1711.

Sandermann, H., 1992. Plant metabolism of xenobiotics. Trends Biochem. Sci. 17, 82–4.

Scandalios, J.G., 2005. Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Braz. J. Med. Biol. Res. 38, 995–1014.

Scheibe, R., Backhausen, J.E., Emmerlich, V., Holtgrefe, S., 2005. Strategies to maintain redox homeostasis during photosynthesis under changing conditions. J. Exp. Bot. 56, 1481–9.

Scheurer, M., Ramil, M., Metcalfe, C.D., Groh, S., Ternes, T. a, 2010. The challenge of analyzing beta-blocker drugs in sludge and wastewater. Anal. Bioanal. Chem. 396, 845–56.

Scheytt, T.J., Mersmann, P., Heberer, T., 2006. Mobility of pharmaceuticals carbamazepine, diclofenac, ibuprofen, and propyphenazone in miscible-displacement experiments. J. Contam. Hydrol. 83, 53–69.

Schoenwaelder, M.E.A., 2002. The occurrence and cellular significance of physodes in brown algae. Phycologia 41, 125–39.

Schröder, P., Maier, H., Debus, R., 2005. Detoxification of herbicides in Phragmites australis. Z. Naturforsch. C. 60, 317–24.

Seiler, J.P., 2002. Pharmacodynamic activity of drugs and ecotoxicology--can the two be connected? Toxicol. Lett. 131, 105–15.

Sensi, N., Schnitzer, M., 1977. Effects of pH, reaction time, chemical reduction and irradiation on Esr spectra of fulvic acid. 123, 224–34.

Sersen, F., Kralova, K., Bumbalova, A., 1998. Action of mercury on the photosynthetic apparatus of spinach chloroplasts. Photosynthetica 35, 551–9.

Shenker, M., Harush, D., Ben-Ari, J., Chefetz, B., 2011. Uptake of carbamazepine by cucumber plants--a case study related to irrigation with reclaimed wastewater. Chemosphere 82, 905–10.

Shi, W., Wang, L., Rousseau, D.P.L., Lens, P.N.L., 2010. Removal of estrone, 17alpha-ethinylestradiol, and 17beta-estradiol in algae and duckweed-based wastewater treatment systems. Environ. Sci. Pollut. Res. Int. 17, 824–33.

Srivastava, A., Guissé, B., Greppin, H., Strasser, R.J., 1997. Regulation of antenna structure and electron transport in PSII of Pisum sativum under elevated temperature probed by the fast polyphasic chlorophyll a fluorescence transient: OKJIP. Biochim. Biophys. Acta 1320, 95–106.

Srivastava, A., Strasser, R.J., 1997. Constructive and destructive actions of light on the, 56th ed. J. Sci. Indust. Res.

Stebbing, a R., 1982. Hormesis--the stimulation of growth by low levels of inhibitors. Sci. Total Environ. 22, 213–34.

St-Pierre, B., Luca De, V., 2000. Evolution of acyltransferase genes: origin and diversification of the BAHD superfamily of acyltransferases involved in secondary metabolism, 1th ed. Elsevier science Ltd, Oxford.

Strasser, B.J., 1997. Donor side capacity of Photosystem II probed by chlorophyll a fluorescence transients. Photosynth. Res. 52, 1997.

Streb, P., Feierabend, J., 1996. Oxidative responses accompanying photoincactivation of catalase in NaCl-treated rye leaves. Bot. Acta 109, 125–32.

Stresser, D.M., Blanchard, A.P., Turner, S.D., Erve, J.C.L., Dandeneau, A.A., Miller, V.P., Crespi, C.L., 2000. Substrate-dependent modulation of CYP3A4 catalytic activity: Analysis of 27 test compounds with four fluorometric substrates. Pharmacology 28, 1440–8.

Stumpf, M., Ternes, T. A., Wilken, R.D., Rodrigues, S. V, Baumann, W., 1999. Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brazil. Sci. Total Environ. 225, 135–41.

Svensson, J., Fick, J., Brandt, I., Brandström, B., 2013. The Synthetic Progestin Levonorgestrel Is a Potent Androgen in the Three-Spined Stickleback (*Gasterosteus aculeatus*). Environ. Sci. Technol. 47, 2043-51.

Taiz, L., Zeiger, E., 1998. Plant Physiology, Sunderland. ed. Sinauer Association.

Ternes, T.A., 1998. Ternes 1998 Occurrence of Drugs in German sewage.pdf. Water Res. 32, 3245–60.

Tixier, C., Singer, H.P., Oellers, S., Müller, S.R., 2003. Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface waters. Environ. Sci. Technol. 37, 1061–8.

Trebst, A., 1972. Measurement of the Hill reaction and photoreduction., Methods in. ed. Academic Press, New York, NY.

Tsimilli-Michael, M., Krüger, G.H.J., Strasser, R.J., 1995. Suboptimality as driving force for adaptation: A study about the correlation of excitation light intensity and the dynamic fluorescence emission in plants., In Mathis,. ed. Kluwer Academic, The Netherlands.

Turgut, C., Fomin, a, 2002. Sensitivity of the rooted macrophyte Myriophyllum aquaticum (Vell.) verdcourt to seventeen pesticides determined on the basis of EC(50). Bull. Environ. Contam. Toxicol. 69, 601–8.

Van Eerd, L.L., Hoagland, R.E., Zablotowicz, R.M., Hall, J.C., 2003. Pesticide metabolism in plants and microorganisms. Weed Sci. 51, 472–95.

Van Rensberg, L., Krüger, G.H.J., Eggenberg, P., Strasser, R.J., 1996. Can screening criteria for drought resistance in Nicotiana tabacum L. be derived from the polyphasic rise of the chlorophyll a fluorescence transient (OJIP)? South African J. Bot. 62, 337–41.

Vernouillet, G., Eullaffroy, P., Lajeunesse, A., Blaise, C., Gagné, F., Juneau, P., 2010. Toxic effects and bioaccumulation of carbamazepine evaluated by biomarkers measured in organisms of different trophic levels. Chemosphere 80, 1062–8.

Viet, U., Moser, E., 2003. Ecotoxicological test assays with macrophytes. Fresenius Environ. Bull. 12, 545–9.

Viglino, L., Aboulfadl, K., Prévost, M., Sauvé, S., 2008. Analysis of natural and synthetic estrogenic endocrine disruptors in environmental waters using online preconcentration coupled with LC-APPI-MS/MS. 76, 18761160.

Voeste, D., Levine, L.H., Levine, H.G., Blum, V., 2003. Pigment composition and concentrations within the plant (Ceratophyllum demersum L.) component of the STS-89 C.E.B.A.S. Mini-Module spaceflight experiment. Adv. Space Res. 31, 211–4.

Voutsas, E., Magoulas, K., Tassios, D., 2002. Prediction of the bioaccumulation of persistent organic pollutants in aquatic food webs. Chemosphere 48, 645–51.

Vulliet, E., Baugros, J.-B., Flament-Waton, M.-M., Grenier-Loustalot, M.-F., 2007. Analytical methods for the determination of selected steroid sex hormones and corticosteriods in wastewater. Anal. Bioanal. Chem. 387, 2143–51.

Vymazal, J., Kröpfelová, L., 2008. Wastewater Treatment in Constructed Wetlands with Horizontal Sub-Surface Flow, Environmen. ed. Springer Netherlands.

Wang, W., Freemark, K., 1995. The use of plants for environmental monitoring and assessment. Ecotoxicol. Environ. Saf. 30, 289–301.

Weigel, S., Berger, U., Jensen, E., Kallenborn, R., Thoresen, H., Hühnerfuss, H., 2004. Determination of selected pharmaceuticals and caffeine in sewage and seawater from Tromsø/Norway with emphasis on ibuprofen and its metabolites. Chemosphere 56, 583–92.

Weir, T.L., Park, S.-W., Vivanco, J.M., 2004. Biochemical and physiological mechanisms mediated by allelochemicals. Curr. Opin. Plant Biol. 7, 472–9.

Weiss, J., Liebert, H.P., Braune, W., 2000. Influence of microcystin-RR on growth and photosynthetic capacity of the duckweed Lemna minor L. J. Appl. Bot. 74, 100–5.

Wiegel, S., Aulinger, a, Brockmeyer, R., Harms, H., Löffler, J., Reincke, H., Schmidt, R., Stachel, B., von Tümpling, W., Wanke, a, 2004. Pharmaceuticals in the river Elbe and its tributaries. Chemosphere 57, 107–26.

Wynne-Edwards, K.E., 2001. Review Evolutionary Biology of Plant Defenses against Herbivory and Their Predictive Implications for Endocrine Disruptor Susceptibility in Vertebrates. Environ. Heal. 109, 443–8.

Yin, Y., Wang, X., Sun, Y., Guo, H., Yin, D., 2008. Bioaccumulation and Oxidative Stress in Submerged Macrophyte Ceratophyllum demersum L . Upon Exposure to Pyrene. Environ. Toxicol. 328–36.

Zar, J.H., 1996. Biostatistical analysis, Third ed. ed. Prntrice - Hall, Inc. New Jersey.

Zeilinger, J., Steger-Hartmann, T., Maser, E., Goller, S., Vonk, R., Länge, R., 2009. Effects of synthetic gestagens on fish 28, 19469587.

Zenker, A., Cicero, M.R., Prestinaci, F., Bottoni, P., Carere, M., 2014. Bioaccumulation and biomagnification potential of pharmaceuticals with a focus to the aquatic environment. J. Environ. Manage. 133, 378–87.

Zhang, W., Zhang, M., Lin, K., Sun, W., Xiong, B., Guo, M., Cui, X., Fu, R., 2012. Eco-toxicological effect of carbamazepine on Scenedesmus obliquus and Chlorella pyrenoidosa. Environ. Toxicol. Pharmacol. 33, 344–52.

Zwiener, C., Seeger, S., Glauner, T., Frimmel, F.H., 2002. Metabolites from the biodegradation of pharmaceutical residues of ibuprofen in biofilm reactors and batch experiments. Anal. Bioanal. Chem. 372, 569–75.

7. Declaration / Selbstständigkeitserklärung

I hereby declare that this dissertation was independently written without any external assistance and that no source other than those cited were used. Furthermore, I declare that this work is not under consideration for submission to another examining institution.

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~Ralph Waldo Emerson~

9. Publications and Conference participation

9.1 Publication

2013/2014

Wrede. J.D., Nuetzmann. G., Kloas. W., Pflugmacher-Lima. S., "Bioaccumulation of pharmaceutically active compounds in the non-target organism macrophyte *Ceratophyllum demersum"*. (Manuscript in review process)

Wrede. J.D., Kloas. W., Pflugmacher-Lima. S., "Effects of different pharmaceutically active compounds in the non-target organism *Ceratophyllum demersum*".
(Manuscript in preparation)

9.2 Conference participation

06/2013, Berlin - Biodiversity and Health

Poster, young scientist workshop presentation

05/2012, Berlin - SETAC world

Poster

05/2011, Milano - SETAC Europa

Poster und presentation (Poster corner)